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Viral haemorrhagic septicaemia in Finnish brackish water fish farms –
Studies on disease surveillance and epidemiology of viral haemorrhagic septicaemia virus

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ACADEMIC DISSERTATION

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Abstract

Viral haemorrhagic septicaemia (VHS) was isolated for the first time in Finland in 2000 from a Finnish brackish water fish farm farming rainbow trout in net pens in the Province of Åland, Baltic Sea. The efforts to eradicate the disease from the Åland islands were not successful. Epidemical factors, needed for VHS management in viral haemorrhagic septicaemia virus (VHSV) positive brackish water fish farms, were studied in a 3-year project, the results of which are presented in this thesis. The study compared the ability of four different surveillance procedures and three diagnostic tests to reveal whether a fish population was infected with VHSV. The programme that was conducted as syndromic surveillance, where the farmers sent in samples for diagnostics if any signs of possible fish disease were noticed, clearly outperformed the other three programmes, which were based on active surveillance. A real-time reverse transcriptase polymerase chain reaction method proved to be at least as sensitive in detecting acute VHSV infections as virus isolation in cell culture, which is considered the gold-standard method for diagnosing VHSV. An ELISA method was used to test fish serums for antibodies against VHSV and was found to be a promising tool in VHSV eradication, particularly for screening populations during the follow-up period, before declaring an area free of infection.

During the epidemics it was a common suspicion wild fish being the most likely source of the reinfections of VHSV in infected fish farms in the restriction area. Wild fish of 17 different species from VHS-positive fish farms were screened for VHSV during 2005-2008. In addition, uninfected wild perch, roach and farmed whitefish were introduced to a fish farm with rainbow trout experiencing a clinical outbreak of VHS. The wild fish did not test positive on any occasion, but whitefish were infected and started to replicate VHSV for a short time. The replication of the virus in whitefish was verified using a new qRT-PCR method that tests separately for positive- and negative-sense viral sequences in infected organ samples.

The presence of VHSV in the environment on fish farms or processing plants farming or handling VHSV-positive fish was studied by testing samples for VHSV from wild blue mussels (*Mytilus edulis*) living in infected fish farms. Sea water and sediment from infected fish farms were also tested for VHSV. Wild uninfected blue mussels were also challenged with VHSV in two different challenge tests. Wastewater from a processing plant was tested before and after disinfection treatment. Blue mussels were not found to be carriers of VHSV on any occasion. Sea water tested positive for VHSV RNA more often during the wintertime when water temperature was close to 0°C and sunlight (UV light) sparse. Most wastewater samples collected before the disinfection treatment were positive for VHSV, but samples collected after disinfection were all negative regarding VHSV RNA. Contacts between the processing plants and the fish farms in the restriction area of VHS were very common during this study. Processing plants are usually the place where fish food and farming equipment are stored, including boats that are used for the daily servicing of the farming localities. According to the results of this study, this contact was considered a major risk for disease spread, especially during the cold part of the year when daylight is also short.

Altogether, this thesis compiles the results of a series of studies targeting factors that could affect the infection pressure of VHSV on disease free fish populations.

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List of original publications

This thesis is based on the following publications:

I Vennerström P, Välimäki E, Lyytikäinen T, Hautaniemi M, Vidgren G, Koski P, Virtala A-M (2017) Viral haemorrhagic septicaemia virus (VHSV Id) infections are detected more consistently using syndromic vs. active surveillance. Dis Aquat Org 126:111-123. https://doi.org/10.3354/dao03161

II Vennerström P, Välimäki E, Hautaniemi M, Lyytikäinen T, Kapiainen S, Vidgren G, Virtala A-M (2018) Wild fish are negligible transmitters of viral haemorrhagic septicaemia virus (VHSV) genotype Id in the VHS restriction zone in Finland. Dis Aquat Org 131: 187–197. https://doi.org/10.3354/dao03301

III Vennerström P, Maunula L, Välimäki E, Virtala A-M (2020) Presence of viral haemorrhagic septicaemia virus (VHSV) in the environment of virus-contaminated fish farms and processing plants. Dis Aquat Org Vol. 138: 145–154. https://doi.org/10.3354/dao03454

The publications are referred to in the text by their roman numerals.

Abbreviations

BF-2	Bluegill fry (cell line)
ca.	circa (Latin), about
CPE	cytopathic effect
Ct	threshold cycle cut-off
DNA	deoxyribonucleic acid
DTU	Technical University of Denmark
EC	European Commission
e.g.	exempli gratia (Latin), for example
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPC	Epithelioma papulosum cyprini (cell line)
EURL	Community reference laboratory for fish diseases
FI	Finland
G	glycoprotein
ICTV	International Committee on Taxonomy of Viruses
i.e.	id est (Latin), that is
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IHN(V)	Infectious haematopoietic necrosis (virus)
IPN(V)	Infectious pancreatic necrosis (virus)
L	RNA polymerase protein
M	matrix protein
MAb	monoclonal antibody
MEM	minimum essential medium
N	nucleoprotein
NV	non-virion protein
OIE	World Organisation for Animal Health (Office International des Epizooties)

ORF	open reading frame
P	phosphoprotein
PCR	polymerase chain reaction
PNT	plaque neutralisation test
PPR	pattern recognition receptors
RNA	ribonucleic acid
qRT-PCR	quantitative real-time polymerase chain reaction
RT-PCR	reversed transcriptase polymerase chain reaction
TLR	Toll-like receptors
TCID50	50% tissue culture infective dose
VHS(V)	Viral haemorrhagic septicaemia (virus)

1 Introduction

Viral haemorrhagic septicaemia is a fish disease that is caused by viral haemorrhagic septicaemia virus (VHSV), a virus belonging to the genus *Novirhabdovirus* of the *Rhabdoviridae* (Walker et al. 2018) family. VHSV is divided into four genotypes I-IV and has been described in more than 80 fish species in both fresh and marine waters of the Northern hemisphere (review by Skall et al. 2005a, Elsayed et al. 2006, Lumsden et al. 2007, Dale et al. 2009, Bain et al. 2010, Gadd et al. 2010, 2011, Kim and Faisal 2010, Emmenegger et al. 2013, Ito and Olesen 2013).

VHS causes heavy losses due to high mortalities in fish farms, especially in rainbow trout farming. The severity of the infection depends on the fish species and virus strain. VHS is a notifiable disease in the European Union. The disease is listed as a non-exotic disease that is prevented and controlled according to *European Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals* (EC 2006).

Finland joined the European Union in 1995 and started an intensive screening of Finnish fish farms for infectious haematopoietic necrosis IHN, spring viremia of carp SVC, infectious pancreatic necrosis IPN, VHS and bacterial kidney disease BKD, to prove freedom from these diseases. After five years of screening, VHS was diagnosed for the first time in spring 2000 at a fish farm producing rainbow trout for consumption in open net pens in the sea area of the Province of Åland (hereafter called Åland), and almost simultaneously at a similar fish farm on the south coast of continental Finland approximately 330 km away (Raja-Halli et al. 2006). Infection with VHSV genotype Id spread rapidly between fish farms in Åland, despite extensive eradicative measures, and in 2001, a restriction area comprising the whole province was established. Movement of live fish, ungutted farmed fish and fish farming equipment including well boats from the restriction area was forbidden. However, eradicative measures in the second area on the south coast were successful, and VHSV has not been isolated there since 2001. In 2003, VHSV spread from Åland to a third area, to fish farms also producing rainbow trout in the municipalities of Uusikaupunki, Pyhäranta and Rauma, on the west coast of continental Finland (Raja-Halli et al. 2006). VHS was successfully eradicated from this third area in the same year and VHSV was not reported there until 2008, when the virus was isolated again in the same area in a fish farm producing rainbow trout for consumption in Pyhämaa. Eradication was repeated and no infection has been recorded since 2008.

The source of the first VHSV infections in Finland in 2000 is still open. There was no known contact between the two fish farms where the first VHS outbreaks were found in 2000. The strongest suspected source of the infection was wild herring. Einer-Jensen et al. (2004) reported in their evolutionary study on isolated VHSV strains that the Finnish isolates from rainbow trout resembled the old Danish isolates. They suggested that the Finnish isolate could have evolved from marine VHSV types similarly to what is believed to have happened in Denmark in the 1930s when the disease first occurred (Einer-Jensen et al. 2004). The diseased fish of the first cases in Finland had not been fed wild herring, but both farms were situated close to harbours for herring trawlers offering close contact with trawled herring.

The fish farming industry in Åland started to improve biosecurity on farms, but keenness to change the infrastructure to a higher biosecurity standard was low, as wild fish were believed to be the source of reinfections. In Åland, new disease outbreaks were often reported, even as soon as 1-2 weeks after fish from a VHS-free area were moved to localities that had been empty of fish for 8–12 months, including the removal and disinfection of all farming equipment (author's own experience).

Nevertheless, surveillance efforts that screened wild herring, sprat, salmonid brood fish and lampreys (*Lampetra fluviatilis*) for VHSV during 2004–2006 on the west coast of Finland in the Baltic Sea remained negative for VHSV Id (Gadd et al. 2010, 2011). Furthermore, the subsequent screening of wild fish in the vicinity of the study farms reported on herein also suggested a lack of VHSV in wild fish (Vennerstöm et al. 2018). Recurrent outbreaks of VHS in Åland were difficult to explain and suspicion about the rationale and effectivity of the surveillance programme was raised, including the diagnostic sensitivity of screening for the presence of VHSV infection. It was suspected that the surveillance programme and methods used were only able to find the 'tip of the iceberg' and that in order to achieve successful eradication, surveillance activities needed to be improved.

This study tested different surveillance strategies of the farmed fish populations and compared different diagnostic tests for detecting if a population is or had been infected by VHSV. The role of wild fish as reservoir of VHSV was studied. The occurrence of VHSV in the environment like blue mussels, sea water, sediment and wastewater in processing plants handling VHS infected fish were also studied.

2 Review of the literature

2.1 Fish farming and monitoring of fish diseases in Finland

Farming of fish has been practised in Finland since the mid-19th century. In the beginning, eggs were collected from wild brood stock along the main salmon rivers and hatched to restock the natural fish resources. Commercial fish farming started in the late 1950s when salmonids were hatched and farmed in ponds. Fish farming became more intensive in the 1960s when rainbow trout (*Oncorhynchus mykiss*) production grew. Rainbow trout, a fish species originating from the Pacific coast of North America, was imported to Europe in the late 19th century, and shortly after this even to Finland (EC 2012, Finnish Fish Farmers' Association 2019).

The number of fish farms increased, reaching its peak in the early 1990s when the environmental authorities started restricting farming activities by making licensing stricter. Fish farming is a highly restricted and regulated activity in Finland, ensuring the well-being of the environment and fish. Today any fish farming activities that on a yearly basis produces more than 2000 kg fish or uses more than 2000 kg fish feed needs to be licensed according to the Environmental Protection Act (Ministry of the Environment 2014). Additional licensing is needed for building water sources according to the Water Act (Ministry of the Environment 2011) and all fish processing activities need to be licensed. Health authorisation is also acquired for the sale or transfer of fish for food, on-growing or restocking from farms or from ponds with natural nutrition (EC 2014).

Fish are farmed for two main reasons in Finland: producing food fish and restocking the sea, rivers and lakes. Fry for food fish production are mainly received from broodfish farmed in inland farms using natural water from lakes and rivers or well water. The hatcheries consist of a hatchery section and a grow-out section for the newly hatched fry. Fry are usually moved to out-door ponds for further growing before they are sold to food fish producers, which are mainly situated in the sea area where food fish are farmed in net pens that are anchored to the bottom or to the nearby shore.

Companies that farm food fish in the sea area have separate localities for farming during summer and winter. The coast of Finland has quite shallow waters and the conditions for farming salmonids are not optimal regarding water temperature and oxygen levels during summertime. Winter localities are often located in shallow bays or by surrounding islands close to the mainland where they are protected from harsh weather conditions in autumn, and especially in winter when the sea may be covered by ice. During summer, water temperatures often rise over 20 °C in the winter localities and fish are moved to the summer localities situated in cooler and deeper waters more optimal for farming rainbow trout. The farming licences also restrict the amount of fish that may be placed in winter localities and order that fish must be moved to summer localities before a certain date. Fish that have reached slaughter size in autumn are often moved as close to the processing plants as possible or transported by well boats directly from the summer localities for processing. Fish that will not attain slaughter size until the next year are often kept in the winter farming localities for the first year.

Processing plants are usually the place where the fish food and farming equipment are stored, including boats for the daily servicing of the farming localities. One food fish producing company in the sea area usually has several farming localities that are scattered around the many islands of the archipelago (see map in figure 1, Vennerström et al. 2017 (I). There are often daily contacts between most of the farming localities and the processing plant. The winter farming localities are also often situated next to the processing plant.

Rainbow trout has been the main farmed species for food production, but in the 2000s the farming of whitefish (*Coregonus lavaretus*), a native fish species, began in the sea and inland waters. In addition, other native fish species are farmed for food mainly in the inland area, like arctic char (*Salvelinus alpinus*), pike perch (*Sander lucioperca*) and sea trout (*Salmo trutta trutta*) at sea. Sturgeon (*Acipenser sturio*) that is originally imported to Finland is also farmed in a few farms in the inland area (Finnish Fish farmers' Association 2014, Finnish Fish Farmers' Association 2017).

In 2018, the production volume of domestic food fish was 14.3 million kg, about 73% of the peak production year of 1991 (19.6 million kg) (Figure 1). About 83% (11.9 million kg) of the farmed food fish are produced in the sea area, of which 60% (approx. 7.1 million kg) are produced in the Åland Islands, a province on the south-west coast of Finland. In 2018, altogether 13.2 million kg of rainbow trout was produced, which is over 90% of the whole of the food fish production in Finland. In addition, 0.8 million kg whitefish was produced (Natural Resources Institute 2019).

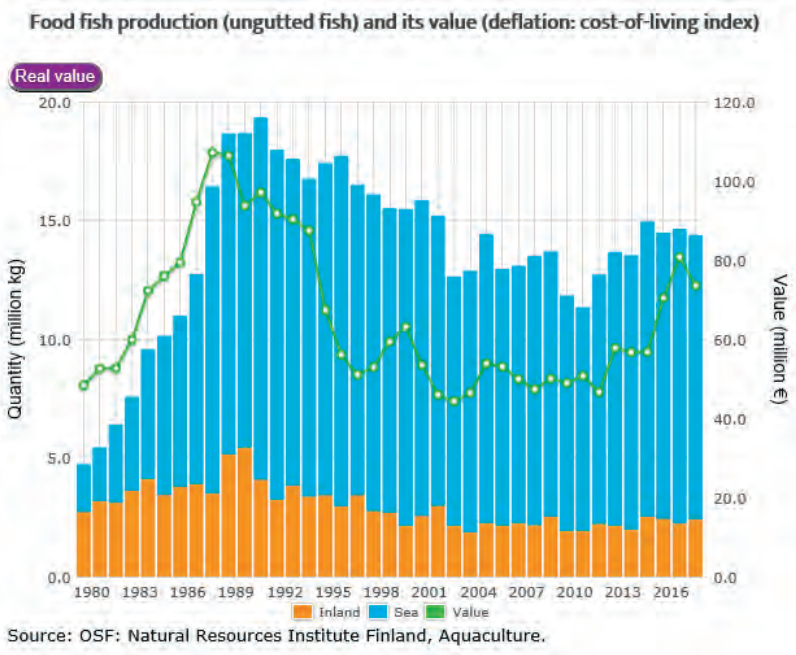

Figure 1. *Annual amount of produced food fish in Finland, 1980-2018 and its value. Figure obtained from Aquaculture Statistics of the Natural Resource Institute Finland (National Resources Institute 2019).*

Restocking of fish has been an important way of ensuring natural resources, especially in waterways where natural spawning migration from the sea is blocked by hydro-power plants. Progeny are collected either from broodfish kept in inland fish farms or from wild-caught broodfish living in either inland water systems or from the sea area. Fertilised eggs are hatched and the fry either moved for further growing in artificial ponds or to a pond with only natural nutrition. Due to the prevention of certain fish diseases spreading from the sea area to inland waters, live fish material (mainly eggs) from the sea may only be brought to the inland area via quarantine. The broodfish, from which the eggs originated, are monitored for certain fish diseases and the eggs are transported into inland waters only if the test results are negative. When fry have gained the desirable size, they are released into the water system to improve the natural resources. Greatest numbers of restocked fingerlings are those of whitefish (*Coregonus lavaretus*) and pike perch (*Sander lucioperca*). The value of the stocked smolts of Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta trutta*) and brown trout (*Salmo trutta lacustris*) is, however, much higher. Some other species like arctic char (*Salvelinus alpinus*) are farmed for this purpose, too.

The prevention of fish diseases in cultivated fish has quite a long history in Finland. A voluntary fish health surveillance programme was established 50 years ago in 1969. This programme was run by the former national veterinary institute (today Finnish Food Authority), has evolved over the years to fit its purpose and is still available for all fish farmers in Finland (Finnish Food Authority 2019). The main aim of the programme has been to ensure that the top of the production pyramid (broodfish and hatcheries) stays as free from fish diseases as possible. This has been well-achieved by categorising fish farms depending on where in the water system they are situated and what kind of production they have. Movement of fish is strictly directed from the top of the pyramid downwards. The laboratory (today the Finnish Food Authority) and the farmer make an agreement about the programme, in which the laboratory agrees to offer diagnostics for fish diseases and give advice to the farmer and local veterinarians regarding health issues on the farm. The farmer agrees to pay an annual fee and inform the laboratory about noticed or suspected fish diseases on the farm by sending samples for diagnostics. The annual fee includes testing of samples in connection to disease issues. The programme has always been voluntary and the coverage of the farms participating in the programme differs depending on type of production. Almost all farms producing progeny (broodfish farms, hatcheries and producers of fry) participate. Among the food fish farmers, the participation has always been quite poor. Screening of notifiable fish diseases was included in the health surveillance programme until Finland joined the European Union (EU) in 1995. After this, screening of notifiable fish diseases was included in the compulsory surveillance programmes issued by the EU. There are no fish health veterinarians working in the field in Finland and the official sampling for screening of notifiable fish diseases is performed by local municipal veterinarians. Although these veterinarians often have minor skills in fish diseases, some gain knowledge in the field during several years of experiences. Three universities perform research on fish health issues, mainly parasitology and bacteriology, and some offer some diagnostic services for fish farms. Fish virus diagnostics has been performed in the Finnish Food Authority national reference laboratory since 1969.

The disease situation in Finnish fish farms has been quite good. In the beginning, bacterial infections like *Vibrio anguillarum* and *Aeromonas salmonicida sp. salmonicida* caused heavy losses and the use of antibiotics was very high (Figure 2). In the mid-1990s, effective vaccines against these two bacterial infections became available and the use of antibiotics in food fish farming decreased dramatically. Today these two bacterial diseases are rare in vaccinated fish. A slight increase in the use of antibiotics was noticed again in 2010-2011 when biotype 2 *Yersinia ruckeri* appeared in food fish farms in the sea area but decreased when vaccination against yersiniosis was started (Figure 2).

Infections with flavobacteria are the main disease problem today and cause high losses in the production of fry, also affecting fish health in the sea area. Commercial vaccines against *Flavobacterium psychrophilum* were available for a few years but were withdrawn from the market as farmers did not use them (author's own experience). The stumbling block for the development of useful vaccines against flavobacterial infections is that the infections are severe and start from newly hatched fry that cannot be vaccinated.

The food fish farming industry faced one of their biggest challenges in 2000 when viral haemorrhagic septicaemia (VHS) was for the first time found in two separate food fish farming areas on the south-west and south coasts of Finland (see section 2.4.2). Another drawback in the disease situation was when IPN was found in 2012 in the inland area that had been IPN-free (Eriksson-Kallio et al. 2016). Eradication of IPN-positive farms was considered impossible and because the IPN isolates were of the less pathogenic genogroup 2, eradication procedures were withdrawn. Inland area is still free from IPN of genogroup 5. A third alerting disease event came in 2017 when infectious haematopoietic necrosis virus (IHNV) was for the first time isolated in sea-reared rainbow trout in two food fish farms on the north-west coast of Finland. In the epidemiological survey of the contact farms, a total of six farming localities were IHNV-positive, two of which were considered possible sources for the other infections. The infected farms have been eradicated and today the infected areas are under surveillance before they can be declared IHN-free compartments again (Finnish food safety authority 2018, Finnish Food Authority 2019).

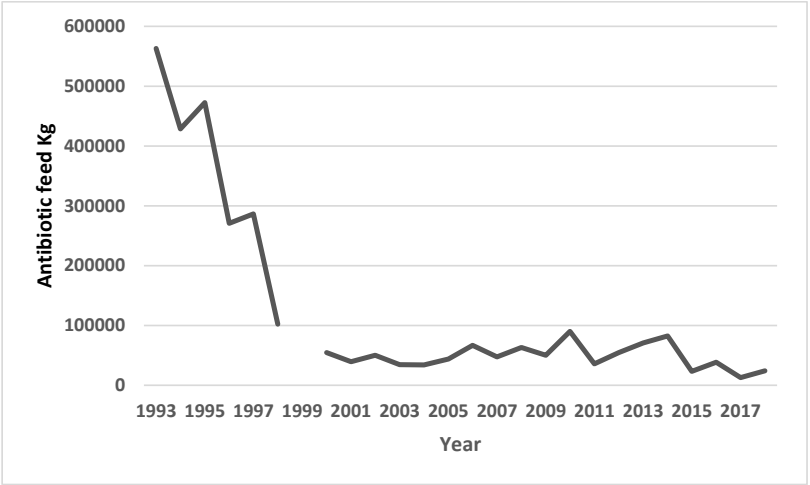

Figure 2. *Amount of produced and imported antibiotic fish feed in Finland 1994-2018. (Information from 1999 is missing)*

2.2 Viral haemorrhagic septicaemia

2.2.1 The disease

In 1938 Schäperclaus described a new serious disease of rainbow trout in Germany that he later termed *kidney swelling and liver degeneration* (Jensen 1965). He described a syndrome with symptoms of acute septicaemia of unknown aetiology that spread among rainbow trout farms and became a serious disease for the trout industry in continental Europe (Schäperclaus, 1954). During winter 1949-1950, a similar syndrome was detected in farms producing rainbow trout in Denmark in a small village called Egtved. Jensen from the state serum laboratory managed to isolate the causative agent for the first time in 1963, a virus that was later confirmed as a rhabdovirus (Jensen 1963, Zwillenberg 1965). The virus was named Egtved virus after the village of the first isolation. Several names had been used for the syndrome among farmers and scientists and Egtved disease became a common name for the disease, although the disease was later named Viral haemorrhagic septicaemia (VHS) by an international agreement in 1963 (Wolf 1988).

The onset of an outbreak of VHS is characterised by nonspecific clinical signs that are followed by rapidly growing mortality. In rainbow trout three different steps of VHS can be seen; acute, chronic and nervous form (Smail 1999). The first disease signs in a VHSV-infected rainbow trout population is that they stop feeding and become lethargic (Wolf 1988). The diseased fish are found close to the edge of the pond or cages where the water is still, and some fish drift against the sieve of the outlet drain as they cannot fight the water current (Wolf 1988). The diseased fish population may get easily scared by movement

above the water surface, making them rush to the edges of the ponds or net pens (Wolf 1988). Diseased fish have a darker pigmentation of the skin, swim erratically and have difficulty in orientation. Bulged eye (exophthalmia) (Figure 3), with haemorrhage around the eye orbit, can be seen in one or both eyes (Wolf 1988). Gills are pale due to severe anaemia (Figure 3). Internally the most pronounced changes can be seen in the kidney and liver (Smail 1999). The kidney is swollen and dark red in the early stages, but later the front and midsection are pale due to necrosis of haematopoietic tissue. The liver is pale and yellowish with areas of mottled haemorrhage and focal areas of necrotic hepatocytes. The most pronounced visible signs in the acute stage are widespread petechial haemorrhages that form as the virus multiplies in the endothelial cells of the capillaries and causes haemorrhages in several location of the body, i.e. the eyes, visceral fat, peritoneum, swim bladder, kidney, liver, skeletal musculature (Figure 3) and heart muscle (Smail 1999). In survivors of the acute stage, haemorrhaging is reduced, but anaemia is severe. Nervous signs are common in the chronic stage: corkscrew-like swimming and some fish may hang in a candle-like position with the head towards the surface and the tail down. The disease signs are not pathognomonic for VHS and may be seen in several other severe septic infections of both viral and bacterial origin. Surviving fish or wild fish that are not sensitive to VHSV are suspected to become carriers of the virus (Schönherz et al. 2013, OIE 2019).

Mortality in VHS-infected rainbow trout varies from mild to severe depending on the virus type, age, stress and water temperature (Smail 1999). VHSV isolates that origin from European freshwater-reared rainbow trout are highly pathogenic to rainbow trout but not to marine fish species (Skall et al. 2004a). Small rainbow trout fry (0.3-3g) have been most susceptible to VHS, as they possess proportionally more of the target organ tissues for the virus than older fish and can reach mortalities up to 80-100% (Smail 1999). Fingerlings and growers are also susceptible, but mortalities are often lower, at 10-50% (Smail 1999). The optimum temperature for virus replication has been estimated from infection trials to be 9-12 °C (Smail 1999, Goodwin and Merry 2011). The highest mortalities are seen in spring when the water temperature is fluctuating. Virus replication is decreased in temperatures over 15 °C (OIE 2019, Avunje et al. 2012). Stress such as handling can reactivate the VHS infection of a population in a carrier state (Hoerlyck et al. 1984, Olesen 1998).

In other farmed fish species like Japanese flounder (*Paralichtus olivaceus*), turbot (*Scopthalmus maximus*) and sea bass (*Dicentrarchus labrax*), symptoms like those in rainbow trout have been described in natural infections of VHSV (Takano 2000, Isshiki 2001). A common characteristic is widespread petechial bleeding in the external and internal organs even though the severity of the bleeding varies in different fish species. VHSV has caused mass mortalities with typical signs of VHS in several wild fish species in North America such as Pacific herring (*Clupea pallasi*) and muskellunge (*Esox masquinongy*). In some species, like cod (*Gadus morhua*), rockling (*Rhinonemus cimbrius*) and haddock (*Melanogrammus aeglefinus*), VHSV has been isolated from skin ulcers (Jensen 1979, Mortensen et al. 1999, Snow 2000, Smail 2000).

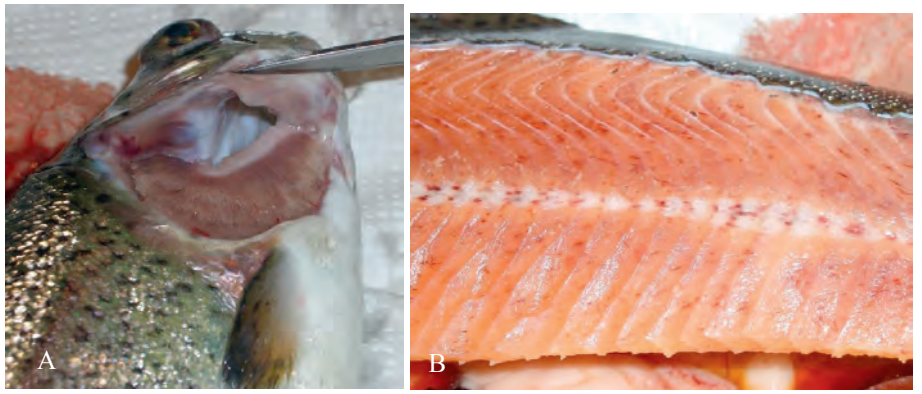

Figure 3. VHS septicaemia in rainbow trout. (A) Exophthalmia and severe anaemia that is seen as pale gills. (B) Petechial bleeding in muscle. (Photo. Sanna Sainmaa)

2.2.2 VHS virus

The causative agent of viral haemorrhagic septicaemia is viral haemorrhagic septicaemia virus (VHSV), which is a *Piscine novirhabdovirus* classified into the genus of *Novirhabdovirus* belonging to the family *Rhabdoviridae* and *Mononegavirales* order (Walker et al. 2018). *Rhabdoviridae* include 18 genera of which 12 infect animals including mammals, birds, reptiles and fish; the remaining 6 genera are arthropod and plant viruses (Walker et al. 2018). One of the most well-known is *Lyssavirus*, which infects several mammals including humans in which they cause fatal encephalitis (rabies) (Walker et al. 2018). Other genera of the *Rhabdoviridae* family that infect fish are *Perhabdovirus* and *Sprivivirus* (Walker et al. 2018). The *Novirhabdovirus* genus include another three species *Hirame novirhabdovirus, Salmon novirhabdovirus* and *Snakehead novirhabdovirus* (Walker et al. 2018). Rhabdoviruses are RNA viruses with bullet-shaped virions (Figure 4) containing a negative-sense, single-stranded RNA (ssRNA) genome (Walker et al. 2018). The virion of VHSV is approximately 70 nm in diameter and 180 nm in length (Kimura et al. 1986, Wolf 1988, Kasornchandra et al. 1992). Like other rhabdoviruses, VHSV has a lipid envelope with glycoprotein (G) anchored to the membrane by a N-terminal hydrophobic transmembrane region. The glycoproteins at the surface of the virion act as the receptor-binding ligand and target of neutralizing antibodies. VHSV isolates can all be identified by the monoclonal antibody (MAb) IP5B11, making the virus serologically homogenous in this respect (Lorenzen et al. 1988). The genomic RNA sequence of VHSV comprises 11,158 bases and contains six genes in the order 3´-*N-P-M-G-NV-L*-5´ (Figure 5). The genome begins with a non-coding 3´leader sequence and terminates with a non-coding 5´trailer sequence. One gene encodes a non-virion protein (NV) (12-14 kDa) (Kurath and Leong 1985, Schutze et al. 1999) and five genes encode structural proteins: the nucleocapsid- (N) (size: 38–47 kDa), phospho- (P) (22–26 kDa, formerly designated M1), matrix- (M) (17–22 kDa, formerly designated M2), glyco- (G) (63–80 kDa) and RNA polymerase protein (L) (150–225 kDa) (Lenoir and de Kinkelin 1975, McAllister and Wagner 1975, Leong and Kurath 2012). The presence of the non-virion gene (*NV*), located between the *G* and *L* genes in the genome, distinguishes members of the genus novirabdovirus from other rhabdoviruses. NV protein is detected in

the nucleus of infected cells but not in virions (Schutze et al. 1999, Choi et al. 2011) The expression of the gene is polycistronic, i.e. each gene is translated from a separate mRNA which are transcribed in a gradient fashion, e.g. more abundant from "left to right" (more mRNA and thus more protein N and least of L) (Maclachlan et al. 2011).

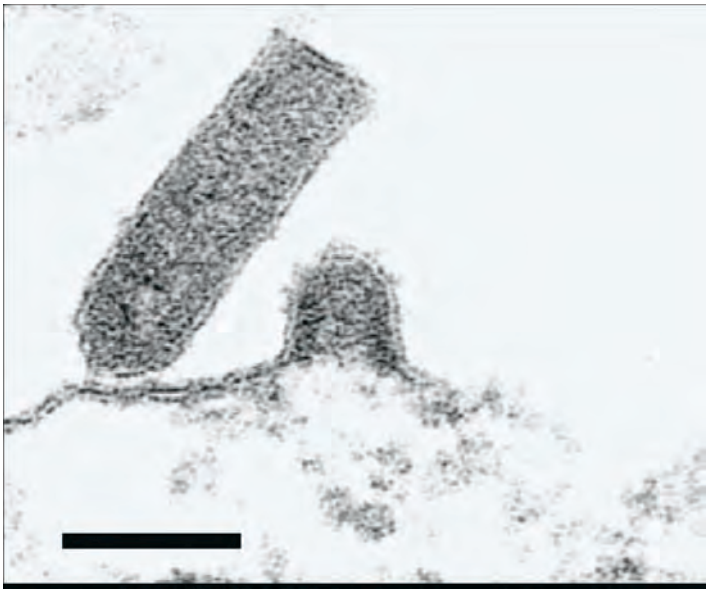

Figure 4. *Electron micrograph showing budding of the fish novirhabdovirus infectious haematopoietic necrosis virus at the plasma membrane and characteristic bullet-shaped rhabdovirus virion structure. Reprinted from Granzow et al. (1997) (Figure 2F) with permission from John Wiley and Sons, Publisher.*

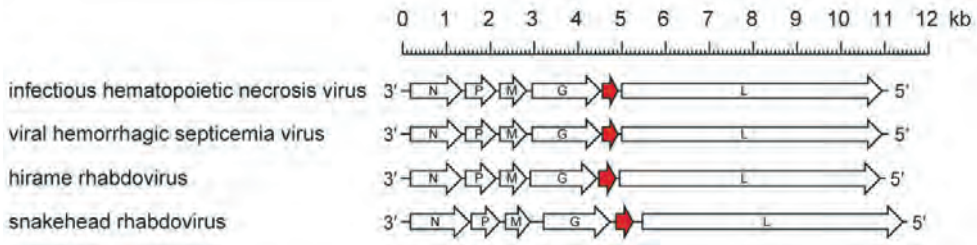

Figure 5. *Schematic representation of novirhabdovirus genome organisations. N, P, M, G and L represent ORFs encoding the structural proteins. ORF NV (U1), encoding a protein involved in pathogenicity and blocking host innate immunity, is highlighted (red). (Reprinted from ICTV 10th report)*

2.2.3 Pathogenesis

To be able to understand the epidemics of VHS, it is important to know how the causative agent manages to develop the disease in its host: (1) survival of the agent in the environment (2) port of entry of the virus to the fish, (3) replication of the virus in the host cell, (4) transmission of the virus, (5) encountering the host´s immune system and (6) escaping the immune system of the fish.

Virus survival outside the host depends on physico-chemical conditions in the environment (Bovo et al. 2005a, 2005b). VHSV is more stable in cold water temperatures (4˚C) than in warm (20˚C). In cold water, VHSV can survive from a few days in natural fresh or sea water and up to a year in filtered freshwater (Parry and Dixon 1997, Hawley and Garver 2008). Freshwater seems to be more favourable for virus survival than sea water according to Hawley and Garver (2008). Rhabdoviruses like VHSV and IHNV are sensitive to UV irradiation (Øye and Rimstad 2001, Yoshimizu et al. 1986).

There are several sequential studies on the pathogenesis of VHS in different fish species experimentally infected by VHSV. These studies indicate that horizontal transmission of virus particles through water is the main transmission route (Stone et al. 1997, Snow et al. 2005, Kurath and Winton 2011). The main entry route into fish challenged by bath is via the gill epithelium and the virus is further transported via the blood to the main internal organs (Smail 1999, Brudeseth et al. 2002a, 2005, Matras et al. 2006). Evensen et al. 1994 found VHSV antigen in rainbow trout endothelial cells of the anterior kidney as early as 2-4 days post-infection. Hepatocytes and exocrine pancreatic cells were also infected less than a week post-infection. The virus starts replicating and causes damage to the endothelium in the circulatory system, as damage in all these organs was noticed to start from day 4. Skin has also been reported as a likely route for virus entry and a site for early replication (Yamamoto et al. 1992). Moreover, an oral infection route has also been reported by feeding infected fish or feed to susceptible hosts such as pike and rainbow trout (Ahne 1980, Meyers 1995, Schönherz et al. 2012).

VHSV replication and transmission

The replication of rhabdoviruses is presented in Figure 6. VHSV enters the host cell by endocytosis that is receptor mediated. The endocytosis is triggered by the viral G protein that is located at the envelope of the virus and binds it to a fibronectin complex (Bearzotti et al. 1995, Assenberg et al. 2010, Purcell et al. 2012). The cell membrane and virus membrane fuse and the viral nucleocapside is released into the cytoplasm of the host cell (Assenberg et al. 2010, Purcell et al. 2012). The infective virion contains an RNA-directed RNA polymerase that transcribes viral genes and uses the host cell to synthesise viral proteins. First a full length single-stranded positive-sense RNA (complementary to the genomic RNA) is produced to generate copies of negative-sense genome RNA for the new virions. The N, L and P proteins are synthesised by free ribosomes and bind to the newly formed viral RNA genome, building up the ribonucleoprotein (RNP) core. The RNP further forms a complex with the M protein. The G protein is translated by ribosomes at the

endoplasmatic reticulum with subsequent posttranslational modifications occurring at endoplasmatic reticulum and Golgi apparatus during the vesicular transport to the plasma membrane of the host cell. The RNP-M complex migrates beneath the plasma membrane enriched with G proteins and promotes budding of enveloped infective virions (Purcell et al. 2012). The virus is spread by urine from diseased fish (Wolff 1988). Vertical spread of VHS virus is not reported and disinfection of eggs with iodine has prevented infection (Olesen and Skall 2013). Significant amount of VHSV may be present in the tissues of processed rainbow trout originating from an infected population and could thereby be transferred to VHS-free areas (Oidtmann et al. 2011a).

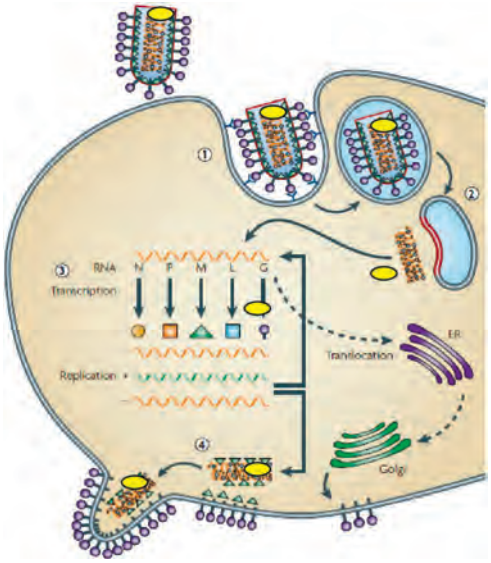

Figure 6. *Virus replication of rhabdoviruses is divided into four main parts. 1 Phagocytosis, virus binds to the cell and enters. 2 Uncoating, viral genome and polymerase is released by fusion of the glycoprotein and matrix membrane. 3 Transcription of a positive-sense RNA that is translated into viral proteins by the endoplasmic reticulum (ER) and Golgi apparatus. 4 The positive-sense RNA is replicated back to a negative-sense RNA and viral components assembled for release of new virions through budding (Picture: reprinted from Schnell et al. 2010) (with permission from Springer Nature)*

Immunity in fish

Immunity is usually divided into two different strategies in fish, as in other vertebrates: innate immunity that is the unspecific 'first-line defence' in infections and specific humoral immunity that builds up a long-term adaptive immunological memory. Innate immunity consists of the physical barrier and cellular response. The cellular responses in innate immunity are well-described in mammals in which pathogenic molecules are recognised by pattern recognition receptors (PRRs) that lead to the activation of immune effector

molecules like the interferon (IFN) system (Kawai et al. 2008). Immunity against viral nucleic acids, including those of rhabdoviruses, is induced by Toll-like receptors (TLR) 3, 7 and 8 or the retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) (Mogensen and Paludan 2005). Teleost fish recognise viral nucleic acids of rhabdoviruses in almost the same pattern as mammals with some exceptions (Zou et al. 2010). They have in addition a set of non-mammalian TLR genes (Rebl et al. 2010, Palti, 2011). Fish also possess genes encoding cytokines, chemokines and other innate effectors for producing type 1 interferons (IFN) that are expressed in several different tissue types and type II IFN that is mainly expressed in haematopoietic tissues and cells (Altman et al. 2003, Long et al. 2004, Long et al. 2006, Robertsen et al. 2003, Sun et al. 2009, Zou et al. 2007, Milev-Milovanovic et al. 2006, Zou et al. 2005, Stein et al. 2007).

The humoral immune response produces neutralising antibodies that are crucial components in immunity against fish rhabdoviruses and the antibody response in teleost fish (reviewed by Lorenzen and LaPatra 1999). Fish possess several immunoglobulin types IgM, IgD, IgZ IgT and a polymeric Ig receptor pLgR that function as an immunoglobulin transporter to mucosal surfaces (Hansen et al. 2005, Danilova et al. 2005, Hordvik et al. 2002, Zhang et al. 2010, Rombout et al. 2014, Bengten et al. 2015,). There are indications that B-cells expressing IgM respond to antigenic stimuli in systemic fish tissues whereas B-cells with IgT function on the mucous membranes (Zhang et al. 2010, Yu et al. 2019). The role of IgT in fish rhabdoviral infections is not known.

It has recently been demonstrated that many of the cells and molecules that have been considered unique to either the innate or adapted immune system actually function in both, making the communication between the systems more complex than earlier believed (Abós et al. 2015, Secombes, 2016).

Cells involved in the immunity of fish are T and B lymphocytes, natural killer cells, monocytes, macrophages, neutrophils, eosinophils, mast cells and thrombocytes (Castro and Tafalla 2015). In addition, dendritic cells have been described in Atlantic salmon (Salmo salar) and rainbow trout (Fuglem et al. 2010, Haugland et al. 2012, Johansson et al. 2012). Fish probably also possess specific cellular immunity, as high levels of specific protection have been recorded in DNA vaccination trials using G protein of novirhabdovirus, although no neutralising antibodies were detected (Lorenzen et al. 1998, LaPatra et al. 2000). Little is known about the role of fish T-cells in infections with fish rhabdoviruses, but some evidence of cell-mediated cytotoxicity (CMC) has been described in host responses to this group of fish viruses (Somamoto et al. 2002, Utke et al. 2008).

Fish are poikilotherm organisms whose physiological functions are slower in water temperatures that are suboptimal for certain fish species. Temperature affects the function of the immune response and virus replication in fish. Immunological suboptimal temperatures lead to an insufficient immunological response due to the slower immune response (Le Morvan et al. 1998). VHSV infections are mostly detected in temperatures below 15 °C, and mortality in low temperatures is usually higher (Wolf 1988). Fish that have survived a VHSV infection clear the virus below the detection level at optimal temperatures and develop good protective immunity. The development of specific immunity is delayed in fish that are kept in low temperatures and the role of innate immunity becomes crucial (Le Morvan et al. 1998, Alcorn et al. 2002, Lorenzen et al. 2009). Low water

temperature is also suggested to be a factor for persistent rhabdoviral infections, probably due to suppression of the humoral adaptive immune response. Neukirch (1986) reported that VHSV was detectable in the brain for 400 days post-infection in rainbow trout held at 4 °C without any clinical signs of infection or detectable antibodies in serum. Another study on VHSV infections in Pacific herring suggested that it is not clear if individuals in a population become carriers of the virus or if the virus persists in the population by jumping between naïve or convalescent hosts (Hershberger 2010, Purcell 2012). Other factors that have been indicative of affecting the immune response are diet, age, seasonality and reproductive status, not to forget stress (Sealey et al 2007, Utke et al. 2008, Beaulaurier et al. 2012, Martin and Król 2017).

Immune evasion of rhabdoviruses

The way in which viruses ensure survival and transmission can be divided into two main strategies: 'hit and run' or 'hit and stay' (Hilleman 2004). Cytolytic viruses like VHSV are mainly considered 'hit and run' viruses as they destroy the infected cell and are highly infective and ready to transmit to new hosts before the host's cell-mediated immunity stops them or the host dies. 'Hit and stay' viruses ensure they can stay in the host by escaping the host´s immune system in a way that ensures virus survival in the host for a longer period, even for good. VHSV may use both strategies depending on the immune status of the host, as varied forms (acute, chronic and nervous) of the disease are well-described. In addition, persistent infection with an asymptomatic carrier state has been described (Vestergård Jørgensen 1982, Neukirch 1986). VHSV is sensitive to the effects of IFN, but virulent rhabdovirus strains can continue replicating in the host despite IFN, as they possess several different mechanisms to evade the host's immune system (Ahmed et al. 1998). They may directly interfere with the interferon system, e.g. the M protein is able to mediate cell shut-off in infectious haematopoietic virus (IHNV) infections (Chiou et al. 2000). Cell shut-off is a cascade of reactions interfering with the programmed cell death process (apoptosis) that host cells use to get rid of virus-infected cells before the pathogen can multiply in the cell (Ahmed et al. 1998, Lyles 2000). Cell shut-off has so far not been described for other fish rhabdoviruses than IHNV (Purcell 2012). The NV protein of Novirhabdoviruses also participates in the immune evasion of the virus by preventing apoptosis and interfering with the unspecific innate immune response of the host (Ammayappan et al. 2011, Kim and Kim 2013, Biacchesi et al. 2017). NV can also depress the IFN response in fish, but indication of this has only been shown for IHNV (Choi et al. 2011). The NV protein is required for effective virus replication and is suspected to be an essential contributor of the pathogenicity of VHSV and IHN (Biacchesi et al. 2017, Johnson et al. 2000, Thoulouze et al. 2004, Ammayappan et al. 2011). Antigenic escape by antigenic drift in the *G* gene (virus evolution) induced by immune selection is not clearly demonstrated, although there are results indicating that it may happen (Huang et al. 1996, Troyer et al. 2000, Kurath et al. 2003, Purcell 2012).

2.3 VHSV strains, hosts and geographical distribution

Viral haemorrhagic septicaemia virus (VHSV) has been detected in more than 80 fish species in both fresh and marine waters in Europe, North America and Asia (Skall et al. 2005a, Elsayed et al. 2006, Lumsden et al. 2007, Dale et al. 2009, Bain et al. 2010, Gadd et al. 2010, 2011, Kim and Faisal 2010, Emmenegger et al. 2013, Ito and Olesen 2013, OIE 2019). VHSV is endemic in fish populations in large areas of the Northern hemisphere (Olesen and Skall 2013). VHSV isolates from both freshwater and the marine environment are divided into four genotypes (later group I-IV) of which I and IV have several sublineages (Ia–Ie, IVa–IVc) (Bernard et al. 1992, Snow et al. 1999, Einer-Jensen et al. 2004, Elsayed et al. 2006, Ammayappan and Vakharia 2009, Pierce and Stepien 2012a, 2012b). The genotypes were formed by sequencing the *N* gene. (Snow et al. 1999, Einer-Jensen et al. 2005, Elsayed et al. 2006, Gagné et al. 2007). Similar virus isolates have also been sequenced by the *G* gene to study genetic evolution, and this resulted in same grouping of the strains (Nishizawa et al. 2002, Einer-Jensen et al. 2004, 2005, Elsayed et al. 2006, Gagné et al. 2007, Gadd et al. 2010, 2011).

VHSV **group I** includes five sublineages (a, b, c, d, e) of which 'a' represents European freshwater isolates and isolates from sea-reared rainbow trout and turbot (Schlotfeldt et al. 1991, Snow et al. 2004, Einer-Jensen et al. 2004, Toplak et al. 2010). VHSV sublineage 'a' can further be divided into two major subpopulations, Ia-1 and Ia-2 (Kahns et al. 2012). VHSV sublineage 'b' strains originate from the Baltic Sea, Skagerrak, Kattegat, the North Sea, and the English Channel and there has been one case in Japan (Snow et al. 2004, Einer-Jensen et al. 2004, Skall et al. 2005b, Nishizava et al. 2002, Nordblom and Norell 2000). Sublineage 'c' includes older Danish freshwater isolates from rainbow trout and has also been reported in Germany and Austria (Jonstrup et al. 2009). Sublineage 'd' includes Scandinavian isolates from the 1960s (Olesen and Skall 2013) and isolates from rainbow trout farms in brackish water in Finland (Raja-Halli et al. 2006). Sublineage 'e' is a single isolate from Georgia (Kalayci et al. 2006, Nishizava et al. 2006, Jonstrup et al. 2009).

Group II includes strains isolated from wild fish in a small region close to Gotland and from the west coast of Finland, mainly herring (*Clupea harengus*) and some isolates from lamprey (*Lampetra fluviatilis*) (Einer-Jensen et al. 2004, Gadd et al. 2010, 2011).

Group III includes isolates from both wild and farmed fish in the North Sea close to the UK and Ireland as well as Norway (Snow et al. 1999, King et al. 2001, Skall et al. 2004b, Dale et al. 2009). The group includes several distinct sublineages that have not been named separately, apart from the Norwegian isolates from 2007 that have been suggested to be named IIIb since they differ genetically from earlier isolated strains (Dale et al. 2009).

Group IV includes three sublineages (a, b, c), of which 'a' consists of North American isolates from wild marine and anadromous fish along the Pacific coast (Meyers and Winton 1995, Marty et al. 1998, Hedrick et al. 2003). Sublineage 'b' is isolates from the Great Lakes watershed of the United States and Canada (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Ammayappan and Vakharia 2009). Strains of sublineage 'c' have been found on the east coast of North America (Pierce and Stepien 2012a, 2012b) Genotype IV isolates have also been found in Japan and Korea (Nishizawa et al. 2002, 2006, Kim et al. 2003, Ito and Olesen 2013). In 2015 VHSV was isolated from wild lumpfish (*Cyclopterus*

lumpus) that was caught to be use as broodfish in Iceland. This isolate differs from earlier reported genotype IV strains and has been suggested to be a novel subgroup for the genotype IV (Guðmundsdóttir et al. 2019)

Rainbow trout (*Oncorhynchus mykiss*) is one of the most sensitive farmed fish species to VHSV genotype I, but turbot is also reported to be sensitive to Ie, III and IVa (Skall et al. 2005b). VHS has also caused severe disease in farmed Japanese flounder (*Paralichthus flesus*) (Isshiki et al. 2001). Extensive mortalities in wild fish due to VHSV genotype IV have been reported in North America in Pacific herring (*Clupea pallasii*) on the Pacific coast and in up to 28 different wild fish species in the Great Lakes watershed and on the east coast (Marty et al. 1998, Meyers et al. 1999, Gagné et al. 2007, Elsayed et al. 2006, Lumsden et al. 2007, Groocock et al. 2007, Ammayappan and Vakharia 2009).

According to phylogenetic studies, VHSV has its ancestors in the marine environment, from where it has adapted to be a serious disease agent for farmed rainbow trout (*Oncorhynchus mykiss*) (Einer-Jensen et al. 2004). The 'change in host range' has probably happened several times since the first reports of clinical outbreaks of viral haemorrhagic septicaemia (VHS) originating from the 1950s (Einer-Jensen et al. 2004). These leaps over species barriers are believed to be a result of human activities in connection to fish farming procedures. Wild marine fish, mainly herring (C*lupea harengus*), were intensively used as minced fresh feed for freshwater farmed rainbow trout in Europe in the 1950s (Meyers and Winton 1995, Dixon 1999, Einer-Jensen et al. 2004). The use of fresh marine fish ceased throughout Europe when several wild marine fish species, including herring, were found to be carriers of VHSV and it was found that it could be spread by the oral route (Stone et al. 1997, Snow et al. 1999, Mortensen et al. 1999). VHSV types pathogenic to rainbow trout have been isolated from wild fish in Europe on several occasions, but mostly no mortality or clinical signs of VHS have been reported in these wild fish species (Skall et al. 2005b).

After VHS was found in marine fish and severe outbreaks in sea-reared turbot were reported from Germany, Scotland and Ireland, wide research programmes to screen wild fish for VHSV were conducted. Screening was performed by Danish, Norwegian and Scottish institutes covering the coastal waters of Scotland, the North Sea, and the coastal waters of the west and south of Norway, Skagerrak, Kattegat and the Baltic Sea (Skall 2005b). They examined a total of 54 137 fish representing 63 different fish species. VHS virus was found in 193 samples from 15 fish species (Mortensen et al. 1999, Smail 2000, King et al. 2001, Brudeseth and Evensen 2002b, Dixon et al. 2003, Skall et al. 2005a). VHSV was found to be endemic in the Baltic Sea, Kattegat, Skagerrak, the North Sea and around the British Isles (Skall 2005a). The prevalence of VHS virus was highest in waters around Bornholm, with prevalence ranging from 0-16.7% for herring and 5.6-7.8% for sprat (*Sprattus sprattus*) (Skall 2005a). Similar prevalence was found in the waters close to California and Oregon in clinically healthy sardine (*Sardinops sagax*) and mackerel (*Scomber japonicus*) (Hedrick et al. 2003)

In Finland, wild salmonid brood fish, Baltic herring (C*lupea harengus membras* L.) and lamprey (*Lampetra fluviatilis*) were screened for VHSV in the coastal waters of the south-west coast of Finland around the area in the Baltic Sea where VHSV-positive (genotype Id) rainbow trout farms are located. VHSV Id was not isolated but Baltic herring and lamprey were found to be carriers of VHSV genotype II (Gadd et al. 2010, 2011). The highest

prevalence of VHS II, 50/479 pools (10.4%), was recorded in herring originating from the Archipelago Sea south-west of Finland (Gadd et al. 2011). However, VHS has never been recorded in farmed fish in this high-prevalence area, even though intensive fish farming has been performed for decades and minced wild herring was earlier used as fresh feed. According to the infection trials, the pathogenicity of the isolated VHSV genotype II strains to rainbow trout was negligible (Gadd et al. 2011).

2.4 Prevention and control of VHSV

VHS is a notifiable disease in the European Union (EU) and one of the diseases listed by the World Organisation for Animal Health (OIE) (OIE 2019). In the EU, VHS is classified as a non-exotic but serious fish disease the spread of which must be prevented according to *European Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals* (EC 2014). In the EU, VHS-free areas have been established by monitoring programmes. Aquaculture areas are classified according to their disease status, and movement of live fish is restricted depending on this status.

Finland received VHS-free status (EC 2005) for the continental areas i.e. freshwater systems, in 2005 after a 10-year period of an EU-approved disease monitoring programme. During this programme, all fish farms were inspected once or twice a year by the competent authority and tested for VHSV every year or every second year depending on the production system. Hundreds of inspection visits and a total of 150,000 samples of farmed and wild fish (mainly salmonids) were screened for VHS during these years (EC 2005).

2.4.1 Diagnosing VHS

Typical clinical signs for VHS are an elevated mortality with severe signs of a septicaemic infection (see section 2.2.1). The visible pathological signs are not pathognomonic for VHS, but a rise in mortality in connection to a water temperature below 15˚C should always raise a suspicion of a serious virus infection and lead to sampling of diseased fish. EU and OIE have given detailed instructions concerning the surveillance and diagnostics of certain diseases of aquatic organisms including VHS (EC 2015, OIE 2019). Briefly, if a suspicion of VHS is raised, the farmer is obliged to inform the authority who inspects the farm and takes a minimum of 10 diseased fish for virological diagnostics. The fish should be packed in a thermo box with coolers, as the fish must be kept cool during transport. Fish should be in the lab for further processing not later than 48 h post-euthanasia. Sampling is then performed depending on the size of the fish. If the fish length is less than 4 cm, the whole fish is minced after the removal of the body posterior to the gut opening. From fish sized 4-6 cm, the viscera including kidneys should be collected. Larger-sized fish should be sampled from the spleen, head kidney, heart and brain. Samples from no more than 5 fish should be pooled in case of clinical suspicion. Samples are homogenised in Eagles minimum essential media (MEM) and kept on ice during the process. The homogenate is centrifuged, and supernatant is tested with real-time RT-PCR for VHSV RNA (Chico et al. 2006, Matejusova et al. 2008, Jonstrup et al. 2013, Hoferer et al. 2019). In addition, a parallel sample is inoculated into BF-2 and EPC cell cultures to gain the virus strain that can be further sequenced (genotyped) if necessary or just confirm the presence of VHSV by a molecular or antibody-based antigen detection method, e.g. ELISA or IFAT. The sending of whole fish samples is justified as one may also rule out other diseases such as bacterial septicaemias, like infections by *Yersinia ruckeri* or *Flavobacterium psychrophilum* that cause symptoms that are difficult to differentiate from VHS and are common in water temperatures below 15 ˚C. It is very important to be able to either confirm or rule out the presence of notifiable diseases as quickly as possible, as the farm is probably under restriction and waiting for results may cause heavy economic losses and mental stress to the farmer during the testing.

Other diagnostic alternatives are also available that could be used when agent detection is not possible. Serological tests may be useful when the water temperature is too high for virus detection. Antibodies against VHSV take 3-4 weeks to appear in the serum after the infection, but high antibody levels may persist for more than 6 moths (Fregrenda-Grandes and Olesen, 2007, Lorenzen and LaPatra 1999). There is no commercial serologic VHSV antibody test available and an evaluation of tests' diagnostic sensitivity, specificity and reproducibility is still needed. Therefore, serology alone is still not recommended to be used in VHSV diagnostics (OIE 2019). Another method to show the presence of VHSV is immunohistochemistry, which could be used to raise a suspicion of VHSV infection but needs confirmation by other methods (Evensen et al.1994).

2.4.2 Epidemics and disease management

VHS has caused severe problems in rainbow trout farming in several countries in Europe due to economic losses and trade restrictions. In Denmark, VHSV hampered the trout industry starting from the early 1960s when approximately half of its 800 farms were considered infected by VHSV (Olesen et al. 2013). The losses due to the disease were serious, as the industry could not in the long run deal with the situation. An eradication programme including stamping-out of positive populations, strict biosecurity, regular clinical inspections with sampling of fish populations on farms and trade regulations between different areas with different disease status were established. The programme was based on voluntary participation and the costs of the programme were paid by the industry. The measures proved to be effective and the number of VHSV-infected farms decreased from 400 to approximately 100 in the first ten years (Olesen 1998). In the early 2000s, the total amount of infected farms was less than 30, all situated in a certain area of rivers with outlets in a brackish water lake on the west coast of Jutland. In 2009, fish farmers and their association endorsed a highly coordinated compulsory eradication programme to get rid of the disease for good. This plan included eradication of all positive farming localities and keeping some high-risk farms empty of fish for two years (brackish water farms) and some for six weeks every spring during a two-year period. The costs of the programme were partly financed by the European Fisheries Fund (EFF), which only compensated the value of the fish and the cost of their removal. Farmers paid the cost of cleaning and disinfecting the farm and bore the loss of income during the fallow. After 50 years of costly and laborious efforts, Denmark was declared VHS-free in 2013 (Olesen et al. 2013).

Other examples of successful eradications are from the UK and Norway. In the UK VHSV infection was detected for the first time in a rainbow trout farm situated in a river system on the east coast, in 2008 (Stone et al. 2008). The disease was eradicated from the farm, and no further spread was noticed in the epidemiological investigation of contacts or other farms in the same river system. The source of the infection was not clear, but one suspicion was raised concerning a fish smokery/processing site upstream that handled imported rainbow trout; infective material may have been released into the river. The isolated VHSV strain was genotype Ia, closely related to freshwater types in continental Europe and highly pathogenic for rainbow trout (Stone et al. 2008).

In Norway, VHSV was isolated from sea-reared rainbow trout with elevated mortality and abnormal swimming behaviour in 2007. The isolated agent was a marine genotype III, a group of strains that had earlier been demonstrated in wild fish in the North Sea but never from rainbow trout. It was known that earlier isolated marine strains, including genotype III, had been almost apathogenic to rainbow trout in infection trials (Skall et al. 2004b). In 2007, VHSV was also detected in an immunohistochemical staining from lesions on the internal organs of the diseased rainbow trout, confirming that this was a clinical VHS outbreak. The isolated VHSV strain was clearly pathogenic for rainbow trout in infection trials. It caused 70% cumulative mortality in an immersion trial and almost 100% by intraperitoneal injections (Dale et al. 2009). The infection was diagnosed in three farming localities in 2007, two new localities in 2008 and one in 2009 (National Veterinary Institute

2010). Heavy eradication procedures were conducted with successful outcomes. No new isolations of VHSV have been made in the area after 2009 (Moldal, 2019).

Examples of VHSV epidemics where the outcomes of eradication procedures were not successful are from Sweden and from the Åland islands in Finland. In 1998, VHSV genotype Ib was detected in diseased sea-reared rainbow trout on the west coast of Sweden. Stamping-out procedures were performed but the infection reoccurred in 2000. VHSV was isolated from herring caught close to rainbow trout farms. The herring isolates were almost identical to the genotype that was isolated from the farmed rainbow trout. It was assumed that the herring during their spawning migration brought the infection repeatedly to the farm and made rainbow trout farming in that area impossible (Nordblom and Norell 2000, Jansson and Vennerström 2014).

Another area where the same genotypes of VHSV have been reported in both wild and farmed fish is British Columbia in Canada (Garver et al. 2013). VHSV genotype IVa has been reported repeatedly in farmed Atlantic salmon since the first isolation in 1995. These isolates have been compared genetically to isolates from wild marine fish species and found to be identical or nearly identical (Garver et al. 2013).

In Finland, VHS was first isolated at a brackish water farm producing rainbow trout in the Province of Åland in May 2000 and a few weeks later at a similar farm at Pyhtää on the south coast, approximately 330 km from the first location (Raja-Halli et al. 2006). The infection spread rapidly to several other farms in both areas (Figure 7). All fish in positive farms in Pyhtää were eradicated during 2001 and new cases of VHS have not occurred in that area since then. On the other hand, in the Province of Åland the disease continued spreading despite eradication measures performed in the farms, which were found positive first. In 2001, eradication measures in Åland were withdrawn as unsuccessful and too expensive. The area was classified as a VHSV-restriction area and no live or ungutted fish or farming equipment could be moved from this area.

In 2003, a third VHS outbreak occurred in a rainbow trout farm on the west coast of Finland in Pyhämaa. Stamping-out was performed and VHSV infection was not detected until 2008, when VHSV was detected in clinically healthy fish from screening samples taken in connection with the processing of the fish.

There was no known contact between the two fish farms in Åland and Pyhtää, where the first VHS outbreaks were found in 2000, and the strongest suspicion of the source of infection was wild herring. The diseased fish had not been fed wild herring, but both farms were situated close to harbours for herring trawlers, so there was close contact with trawled herring. The source of the first VHS virus infections in Finland is still open, but it is possible that marine VHSV mutated, or a new strain occurred in Finland. Einer-Jensen et al. (2004) reported in their evolutionary study that the Finnish isolates from rainbow trout resemble the old Danish isolates. They suggest that the Finnish isolate could have evolved from marine VHSV types similarly to what is believed to have happened in Denmark decades ago.

The farm in the third VHS-positive area (Pyhämaa) was known to have had contact with a fish farm in the VHSV-restriction area in the Province of Åland in 2003 and that was the most probable source of the first infection. The source of the second infection in 2008 was not clear. One possible reason could have been a massive escape earlier the same year of a

VHSV-infected rainbow trout population from a farm situated in the part of the restriction area in the Province of Åland that lies approximately 50 km from Pyhämaa (author's own observations). All the studied VHSV isolates from rainbow trout in these three different areas in Finland have been of the same genotype Id (99.3-100% nucleotide identity); wild fish e.g. herring and lamprey in the sea area surrounding the restricted areas have all been genotype II (Raja-Halli et al. 2006, Gadd et al. 2010, 2011). There are no reports of genotype Id being isolated from wild fish.

The three VHS-restriction areas in Finland received an EU-approved VHS-eradication programme in 2003 (EC 2003). Two of the three restriction areas were declared VHSV-free after an intensified screening period according to the demands set by the European Commission, Pyhtää in 2005 and Pyhämaa in 2011 (Figure 7). Eradication measures in Åland have not been successful. Reinfections have occurred, although some farms have been emptied of fish, all equipment disinfected, and the farms kept empty for several months before new fingerlings were transferred from the disease-free area. These setbacks reduced the eagerness of farmers to participate in eradicating the disease from Åland. The appearance of VHS in the magnitude present back in the early 2000s was considered a significant threat to the disease-free status of the continental area (Lyytikäinen et al. 2007).

Strict biosecurity on fish farms and effective disease monitoring to detect infections as soon as possible is important in the control of most fish diseases, including VHS. In Norway, routine clinical inspections performed by authorized veterinarians and fish health biologists has proven to be a good tool in surveillance for freedom from VHS in marine farmed salmonids (Lyngstad et al. 2016). If infection occurs, movement of fish material should be restricted, as infected fish or eggs are the main carries for spreading the disease (Wolf 1988, Oidtmann et al. 2011a, 2011b, Reichert et al. 2013, Bang-Jensen et al. 2014). Un-disinfected farming equipment, boats and personnel may also spread the disease to uninfected farms (Bovo et al. 2005a). In addition, infection is spread via outlet water and escapees from infected farms, and via birds (Olesen and Vestergård Jørgensen 1982).

No effective vaccine is on the market and the most effective control is still avoidance. DNA vaccines against VHS seem to be the most promising (Dalmo 2018).

Year	Pos.farms/Zone
2000	5/ A+B
2001	6/ A+B
2002	14/ A
2003	3/ A+C
2004	2/ A
2005	9/ A
2006	10/ A
2007	2/ A
2008	4/ A+C
2009	6/ A
2010	1/ A
2011	2/ A
2012	1/A
2013-2019	0

Figure 7. *Amount of VHS cases in the three restriction areas A (Åland), B (Pyhtää) and C (Pyhämaa) regarding VHS during 2000-2019. *Free since 2008 **Free since 2011*

3. Aims of the study

The overall aim of the study was to gain knowledge of the epidemiological factors needed for VHS management in VHSV-positive brackish water fish farms farming rainbow trout in net pens in Finland. The specific objectives were as follows:

1. To test the ability of different surveillance procedures and diagnostic methods to find VHSV-infected populations (I)
2. To test if wild fish living in the close vicinity of VHSV-positive rainbow trout populations are carriers of VHSV (II)
3. To study if blue mussels could be carriers of VHSV (III)
4. To study if VHSV can be found in the environment where VHSV-positive populations have been farmed or are handled for processing (III)

4. Materials and methods

Samples that were included and tested in this study were collected from wild fish, farmed fish, infection trials using wild fish, farmed whitefish and wild blue mussels. Samples were also collected from the environment of fish farms with VHS-infected rainbow trout populations and from processing plants handling VHSV-infected fish. This study received ethics permission for sampling and testing fish dno. ESLH-2006-08289/Ym23. A summary of the design of the different studies performed is presented in table 1.

Table 1 *Design of different studies performed*

Publication	Description of study	Study type	Sampling frequency
I	Syndromic surveillance, farmed rainbow trout	Field study	Always if any sign of disease
I	Active random, farmed rainbow trout	Field study	Once every spring and autumn, from arrival at the farm until slaughter
I	Active non-random, farmed rainbow trout	Field study	Once every spring and autumn, from the first VHSV infection noticed until slaughter
I	EU reference, farmed rainbow trout	Field study	Once a year in uninfected farms; every second year in infected farms
II	Screening of wild fish in VHSV-positive farms	Field study	Every spring and autumn during the study period of 4 years
II	Infection trial with wild fish	Field study	Days 0, 10, 14, 21 and 35 post-infection
II	Infection trial with farmed whitefish	Field study	Days 0, 10, 14, 21 and 35 post-infection
III	Screening of wild blue mussels in VHSV-positive farms	Field study	At least once every spring and autumn for two years
III	Infection trial with blue mussels using cultured virus	In vitro	Days 0, 1, 2, 3 and 6 post-infection
III	Infection trial with blue mussels using VHSV-infected rainbow trout	Field study/In vitro	Days 0, 1, 2, 3, 4, 6, 8, 11, 14, 22, 27 and 29 post-infection
III	Testing of sea water for VHSV	Field study	Springtime and wintertime at VHSV-positive fish farms
III	Testing of wastewater for VHSV in a processing plant	Field study	Twice at a plant processing fish from VHSV-positive fish farms

4.1 Description of the study area (I, II, III)

The study was performed on VHS-positive fish farms farming rainbow trout for consumption in net pens, and processing plants handling VHSV-infected fish populations in an area that has been placed under restrictions regarding VHS since 2000. Another farm situated outside the restriction area, but in similar conditions on the coast of the mainland, was used as a control farm in the studies reported in article II. The fish farms in the study areas received their fry from Finnish inland farms that use natural water from lakes and rivers or well water. Fish were also transported from farms situated on the coastal area outside the VHS-restriction area. VHS has never been reported in the inland area or the earlier-mentioned coastal area where fish farming has been continuously screened for fish disease including viral diseases since 1969. Fry had also been imported to the study area, including farm A of this study, from officially VHS-free farms in Denmark before and during this study.

The net pens in the study areas are anchored to the bottom or to the nearby shore. The study area consists of several small islands. The fish farms are scattered around them and can mostly be reached only by boat. The environmental conditions set demands for fish farming in the study area. Fish farming is performed in separate localities during summer and winter. Winter localities are often located in shallow bays and by surrounding islands close to the mainland. Therefrom they are easily reached and protected from harsh weather conditions in autumn and especially during winter when the sea may get an ice cover. During summer, water temperatures often rise over 20 °C in the winter localities and fish are moved to the summer localities situated offshore with the cooler and deeper waters more optimal for farming salmonids. The farming licences restrict the amount of fish that may be placed in winter localities and order that fish must be moved to summer localities before a certain date. Fish that have achieved slaughter size in autumn, are often moved as close to the processing plants as possible or are transported by well boats directly from the summer localities for processing. Fish that will not attain slaughter size until the next year are often kept in winter farming localities for one year and then transported to a summer locality. These winter localities are also often situated next to the processing plant. The processing plant is also the main storage for fish food and farming equipment. The dock of the processing is the home port for the boats that are used for the daily servicing of the farming localities.

The fish farms in the study area have a high number of different wild fish species living in the close vicinity of the net pens. The presence of the different fish species depends on their migrating behaviour: some are long-distance ‘travellers’ like sea trout and herring, whereas perch only migrate to nearby shore or bays.

At the beginning of the study there were five fish farming companies in the VHS-restriction area, each operating on several farms or farming localities. Most of the fish farms in the restriction area had experienced an outbreak of VHS in their fish populations before this study was performed.

4.2 Screening of farmed fish populations, wild fish and blue mussels for the presence of VHSV (I, II, III)

Fish populations from the fish farms of two enterprises in the restriction area, later called farm A and farm B, were screened for the presence of VHSV using four programmes (table 2) (I). The first programme was based on syndromic surveillance, where the farmers were asked to submit 5-10 affected fish to the laboratory for autopsy and diagnostics every time they noticed any signs of disease in their fish populations. The second programme was an active targeted surveillance where the presence of VHSV was tested from three rainbow trout populations, one from farm A and two from farm B. In the third programme, screening of VHSV was started in two rainbow trout populations after a VHS outbreak was reported and additionally two other populations of rainbow trout from the same locality where signs of VHS were not noticed. The fourth programme was the official screening programme of the VHS-restriction area. Serum samples to detect antibodies against VHSV were collected from the fish sampled in programmes 2 and 3.

Wild fish were caught using special designed nets with four different mesh sizes to be tested for VHSV during a period of four years in the VHS-restriction area (II). The fish were caught in the close vicinity of the fish farms of enterprises A and B with VHSV-positive fish populations. Blue mussels were collected from the anchor ropes of the net pens and the surface of the net pens of fish farms with VHSV-positive fish populations (III).

4.3 Infection trials

4.3.1 Wild fish and farmed whitefish (II)

Wild perch and roach were caught with bow nets in the coastal area outside the VHS-restriction area to be used in a challenge test at a VHSV-positive fish farm. The live-caught fish were transported in aerated tanks to the VHS-positive fish farm A and placed into small net pens to be tested if they were infected by VHSV from rainbow trout experiencing a clinical outbreak of VHS (Figure 8). The wild fish were sampled for VHSV testing before transport to the restriction area. Likewise, farmed whitefish were transported from the VHS-free inland area to test if they could be infected by VHSV. Control groups of all three fish species were placed in similar cages and conditions at control farm C situated outside the VHS-restriction area.

Figure 8 *Wild perch and roach were challenged in small net pens that were placed between net pens with clinically VHS-diseased rainbow trout. (Photo: Pia Vennerström)*

4.3.2 Blue mussels (III)

Mussels for two different bath challenges were collected from the anchor ropes of control farm C and transported in cooled boxes on wet paper to be used in two bath challenges with VHSV (Figure 9). The collected mussels were sampled for the presence of VHSV before the challenge started.

In the first bath challenge, two groups (group I and II) of 60 blue mussels each were placed into separate aerated aquariums with sea water collected from control farm C. The trial was performed in the cool room of the autopsy facilities at the Finnish Food Safety Authority (today the Finnish Food Authority) (Figure 10). The mussels were exposed to a suspension of VHSV strain Fika422, genotype Id (GenBank accession no. AY546615), 5 ml each, collected from a cell culture that was added to the aquarium water of both test groups I and II. Group I was exposed for 6 h and group II for 1 day. The third aquarium functioned as a control group and was not exposed to VHSV. At the end of the exposure, the aquarium water of all three aquariums was changed and clean sea water was added. The vividness (actively filtrating water and closing shell by physical touch) of the blue mussels was confirmed before every sampling and change of water, to ensure that the mussels were filtrating water. The mussels were screened for 6 days.

The second bath challenge was performed by placing four rainbow trout infected by VHSV in two aerated aquariums containing 100 mussels each. Two rainbow trout were placed in each aquarium. The exposure lasted for 10 min in group I and 20 min in group II thereafter the rainbow trout were removed from the bath challenge, euthanised, autopsied and their organ samples collected for testing of VHSV individually. The bath challenge of the mussels continued for 4h after the rainbow trout had been removed. After the challenge, the mussels were transported in a cooled box on moist paper to the facilities where the first

challenge trial was performed. The challenged blue mussels were placed in two aquariums, treated and sampled as in the first bath challenge, but the follow-up continued for 29 days. The second trial also included a control group, as in the first trial. The sampling scheme for both bath challenges is described in table 5.

Figure 9. *Wild blue mussels were collected from the anchor ropes of the net pens that are covered by a thick layer of blue mussels. (Photo: Pia Vennerström)*

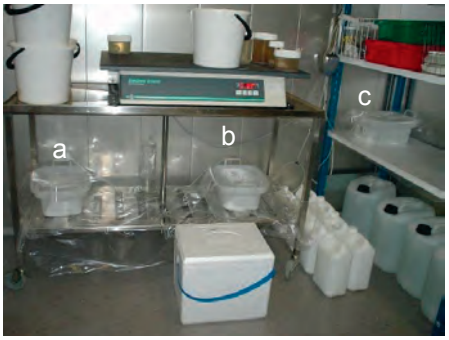

Figure 10. *Blue mussels were challenged in small aerated aquariums, a, b test aquariums and c control group. (Photo: Pia Vennerström)*

4.4 Sampling and treatment of sea water, wastewater and sediment (III)

To test if VHSV could be detected from sea water close to rainbow trout populations experiencing a clinical outbreak of VHS, sea water was collected from the surface and from 2 m depth of fish farms A and B during a clinical outbreak of VHS. Sea water was also collected next to the loading dock of a processing plant processing VHSV-infected fish populations. Sediment was collected from the bottom of fish farm A with VHSV-positive fish populations.

To test if VHSV could be detected in wastewater from the processing line of a processing plant handling VHSV-positive fish populations, samples were collected from the wastewater of different parts of the processing line; CO_2 stunning basin, bleeding basin, kidney remover, drain before liquid waste disinfection and drain after disinfection.

Water samples were treated with methods described by Maunula et al. (2012) and tested at the University of Helsinki, Faculty of Veterinary Medicine, Department of Food Hygiene and Environmental Health. Water samples were prefiltered through a Waterra® filter FHT-700, Waterra) (Anon 2000) from which virus particles were eluted using 50 ml of 50 mM glycine-3% beef extract. The filtration continued through a GF/F membrane and virus eluted from this filter by 1 ml AVL lysis buffer.

Aquarium water from the infection trials and the wastewater samples was not filtered before RNA extraction. Eluates from both filters and unfiltered aquarium water were subjected to RNA extraction by viral RNA Mini Kit (Qiagen).

Sediment samples were diluted by taking 5 g of each sample and adding 1 mL of PBS. RNA extraction from the diluted and stirred sediment samples was performed using a NucliSens magnetic extraction kit (Biomérieux, Boxtel, Netherlands).

4.5 Diagnosing VHSV

4.5.1 Virus detection by virus isolation and qRT-PCR (I, II, III)

The intention of this study was not to find new diagnostic methods, but to use methods that had already been tested and found to be useful and reliable. Virus isolation in cell cultures is considered the gold-standard method for detecting VHSV and was used in this study for all samples except some samples taken from sea water and wastewater. Virus isolation is laborious and time-consuming and therefore we tested three molecular methods, conventional RT-PCR, nested PCR and qRT-PCR, to find a method suitable for detecting VHSV in this study. A qRT-PCR method earlier described by Chico et al. (2006) with minor modifications was chosen to be tested as the most appropriate PCR method. qRT-PCR was used in parallel with cell culture from the same suspensions used for cell culture except for some of the water and wastewater samples that were only tested by qRT-PCR (I, II, III).

Virus isolation in cell culture (I, II, III)

Tissue samples of the brain, anterior kidney and spleen from fish and hepatopancreas from blue mussels were processed according to standard virological procedures (EC 2006, EC 2015, OIE 2019). Organ samples of a maximum of 5 fish or blue mussels were pooled, homogenised and centrifuged (4000 × *g*, 15 min). Samples from sea water, aquarium water from infection trials and wastewater were only stirred before centrifuging. The supernatant was collected for immediate inoculation into 24-well tissue culture plates (Nunc) with monolayer cell cultures of bluegill fry fibroblasts (BF-2) or epithelioma papulosum cyprinid (EPC) epithelial cells (Olesen and Vestergård Jørgensen 1992). The samples were inoculated in BF-2 and EPC cells no later than 24 h post-euthanasia except for programme 4 (official screening in article I), where the instructions given in Commission Decision 2001/183/EC (EC 2001) were followed. The maximum time between euthanasia of the fish and inoculation of samples into cell culture was 48 h in the last-mentioned method.

Real-time RT-PCR for examining the presence of VHSV from tissue suspensions, cell culture, sea water, aquarium water, wastewater and sediment (I-III)

A volume of 1 ml of the same organ suspension and water samples that were used for virus isolation was frozen at –80°C for real-time RT-PCR. RNA extraction was carried out using RNeasy Mini Kit (Qiagen) starting with 200 µl suspension according to the manufacturer's protocol, and the final elution volume was 32 µl.

RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Five microliters of extracted RNA was used in a 25 µl reaction volume. The final concentrations of the primers and the probe were 300 and 100 nM, respectively. The RT reaction profile was: 30 min at 50°C, 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

The primers and the probe for the real-time RT-PCR were manufactured (MedProbe) according to the VHSV sequence from GenBank accession no. D00687 after Chico et al. (2006). The probe was 5-end labelled with FAM fluorescent dye and 3-end labelled with TAMRA fluorescent dye.

To test the sensitivity and repeatability of the qRT-PCR method used, we made a dilution series of VHSV strain Fi-ka422, AY546615 (Einer-Jensen et al. 2004). The dilution series was run several times to test the repeatability of the test and to estimate the threshold cycle (Ct) cut-off (Figure 11). The virus titre was estimated using Spearman-Kärber's method (Mahy and Kangro 1996), estimating 50% tissue culture infective dose (TCID50/ml).

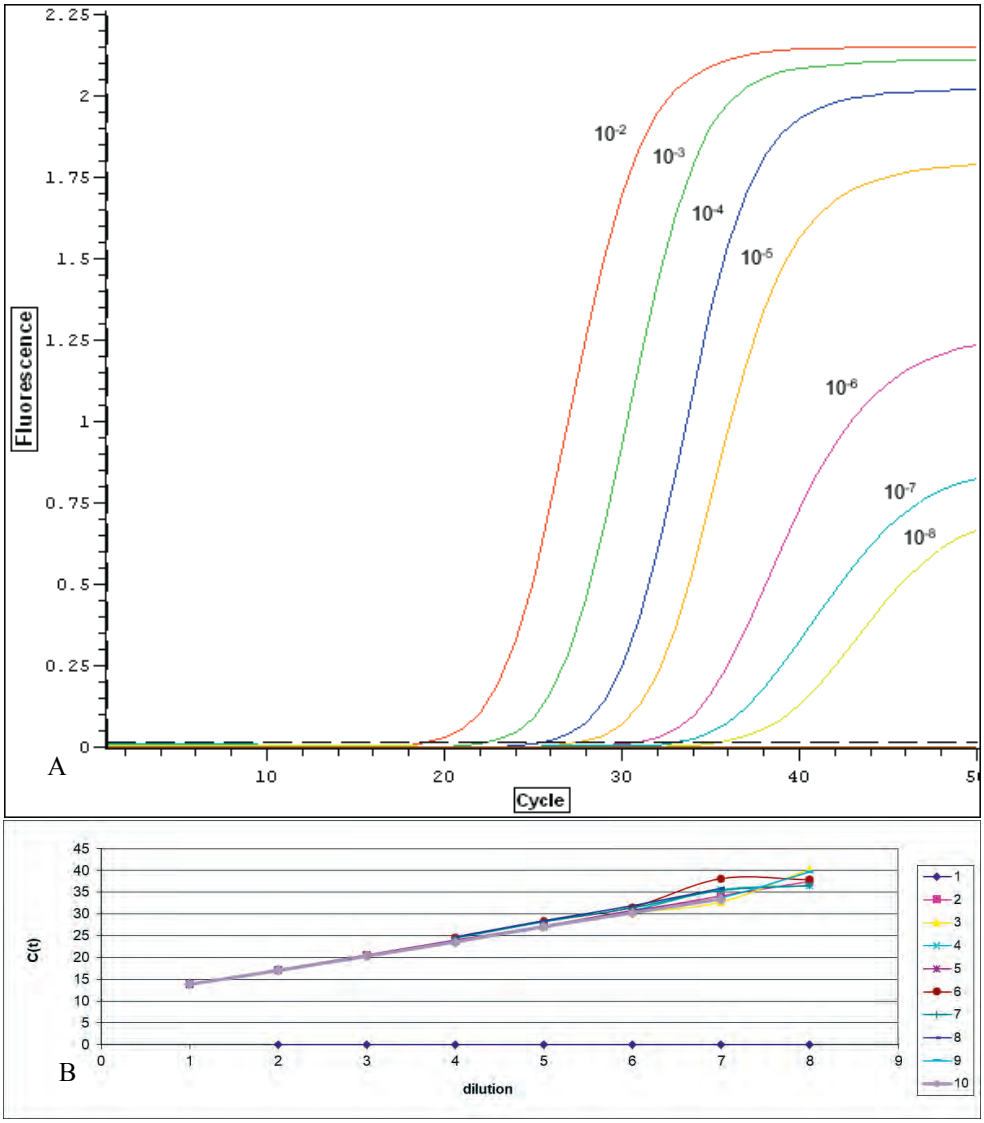

Figure 11. *A: The qRT-PCR curves of 10-fold dilutions (10^{-2}-10^{-8}) of VHSV strain Fi-Ka422, AY546615. B: The threshold cycle of repeated qRT-PCR of dilution series of Fi-Ka422.*

4.5.2 Strand specific qRT-PCR (II, III)

The sea water of the fish farms with VHSV-infected rainbow trout was assumed to be highly contaminated by VHSV during the study. Several of the sampling occasions were even performed during clinical outbreaks of VHS, and a way to prove that the positively tested

tissue samples were a result of virus infection and not contamination was needed. For this purpose, a new strand-specific qRT-PCR method was developed to test for negative- and positive-stranded VHSV products formed during VHSV replication in fish cells. This strand-specific qRT-PCR method is based on a method previously described by Purcell et al. (2006), who used it to test for replication products of infectious haematopoietic virus (IHNV) in salmon.

Controls for the strand-specific method were created with *in vitro* transcription from cloned VHSV *N* gene amplicons. To create a template for positive and negative control RNA, an 810 bp amplicon from VHSV *N* gene PCR was inserted into a pSC-A plasmid and transfected into StrataClone SoloPack competent cells (StrataClone™ PCR Cloning Kit, Stratagene) according to the manufacturer's instructions. The resulting plasmids were purified using QIAprep Spin Mini – prep Kit (Qiagen) and verified by restriction digestion and sequencing with the universal T3 and T7 primers. Sequencing was performed at the Institute of Biotechnology, University of Helsinki, Finland.

The control RNA for strand-specific qRT-PCR was prepared in 2 separate *in vitro* transcription reactions to produce both positive- and negative-strand RNA.

Based on sequencing, the orientation of the insert could be determined, and linearization of the plasmid was performed with *Bam*HI (Fermentas) or *Hin*dIII (Fermentas) for positive- or negative-strand RNA transcription, respectively. *In vitro* transcription of linearized plasmids was performed using MAXIscript T7/T3 Transcription Kit (Ambion, Applied Biosystems) according to the manufacturer's instructions. Both the positive- and negative-strand RNA concentrations were adjusted to 200 ng μl^{-1}, and the RNA was aliquoted and stored at −70°C.

For the detection of positive-stranded RNA, 0.5−2 µg of total RNA was isolated from fish organ pools using RNeasy Mini Kit. Reverse transcription was performed according to Purcell et al. (2006) and Chico et al. (2006). Tagged antisense primer (reverse) was used to synthesise positive-strand based cDNA and tagged sense primer (forward) to synthesise negative-strand based cDNA (Vennerström et al. 2018).

4.5.3 Serology (I)

Serum samples from rainbow trout from farms with VHSV-infected populations were tested for antibodies against VHSV. Serology may reveal VHSV-infected fish populations that have been missed in screening for the virus.

The collected blood samples were centrifuged (3000 × *g*, 15 min) to obtain serum. The serum samples were heat-inactivated for 30 min at 45°C (Olesen et al. 1991) and frozen (–80°C) until examination. The serum samples were tested for the presence of VHSV antibodies using an indirect ELISA method (diagnostic specificity, Sp: 1.0; diagnostic sensitivity, Se: 0.92) (Olesen et al. 1991). The ELISA results of this study were verified by sending a set of samples to be tested in parallel in another laboratory with experience of VHSV serology. This was performed because there have been reports that the results may depend on the used virus strain (antigen) and unspecific factors in the tested serum itself (Fregrenda-Grandes and Olesen 2007). The laboratory was in France at

Ploufragan/Plouzané, Unité de pathologie virale des poissons (Afssa). The samples were tested with the same ELISA method and with a serum neutralisation test (diagnostic specificity Sp 1.0 and sensitivity Se 0.6) (Olesen and Vestergård Jørgensen 1986, Olesen et al. 1991, Castric et al. 2009). The received results were consistent with our results.

4.6 Statistical analyses (I, II, III)

To test objective 1 of this study, the effectiveness of different surveillance programmes in detecting VHSV infections was estimated using a binomial generalised linear model (GLM) (logit link): Logit(Y) = a(Programme1) + b(Programme2) + c(Programme3) + d(Programme4) + $eT + fT$ 2 where Y = a positive detection of VHSV, T = temperature (°C) and a, b, c, d, e and f = coefficients. Programme 4 was treated as a reference category and was the intercept of the estimated model. An omni-bus test was used to determine whether the model was better than the intercept-only model. A model without temperature as a covariate was estimated to assess whether the inclusion of temperature changed the relative efficiencies of the programmes. Probabilities of detection were calculated from a logistic model in the usual way: Probability of detection = e(relevant part of the GLM)/[1+e(relevant part of the GLM)]. For more information, see Dohoo et al. (2009). Statistical analyses were performed using IBM SPSS Statistics version 22.

The modified qRT-PCR test was compared with virus isolation by cell culture (gold-standard test for detecting VHSV) in order to calculate the diagnostic Se and Sp using EpiTools (Sergeant 2016). The threshold cycle (Ct) cut-off was estimated using 2-graph receiver operating characteristic (TG-ROC) curves (Caraguel et al. 2011) with EpiTools (Sergeant 2016).

In objective 2 of this study, the 95% confidence intervals for observed proportions between different wild fish species were calculated using EpiTools (Sergeant 2016) using Jeffrey's method (Brown et al. 2001). The median prevalence estimate (%) was calculated using R (R Core Team 2016).

Objectives 3 and 4 were descriptive studies where the 95% confidence intervals (CI) for percentages were calculated similarly as for objective 2 using Wilson's method.

5. Results

5.1 Surveillance programmes and diagnostic methods (I)

The sampling scheme of the different surveillance programmes are presented in table 2 and results in figure 12. VHSV was detected by virus isolation in 75% of the 12 sampling occasions, when tested from fish showing signs of disease that were sent in for autopsy by fish farmers (programme 1). This procedure gave up to a 17 times higher probability of detection of VHSV than the official screening procedure (programme 4). Programme 2, designed as the official programme 4 but including more frequent sampling of the same fish population than the official programme, gave a result that did not differ from the official programme 4 (Generalised Linear Model, GLM, $P>0.05$). When screening was performed in fish populations that were known to have been VHSV-positive when screening started, the probability of detecting VHSV was 7.7-8.3 times higher than when the official programme 4 was used (GLM, $P<0.05$).

Antibodies against VHSV were detected in only 4 serum samples out of 120 in programme 2. In programme 3, where the tested populations had experienced a clinical outbreak just before the start of the surveillance, all populations were positive on several occasions (I, Table 2).

qRT-PCR, with cut-off set at Ct value of 36, corresponded well with the virus isolation on the separate sampling occasions in programmes 1-3 (kappa value 0.877). The diagnostic sensitivity and specificity of qRT-PCR was 1 and 0.959 respectively when using virus isolation in cell culture as the gold-standard test for detecting VHSV.

Table 2 *Code of action in different surveillance programmes. N number, qRT-PCR real-time reverse transcriptase PCR.*

Action	Program 1 Passive	Program 2 Active/random	Program 3 Active non-random	Program 4 EU-reference
Sampling times	always if mortality elevated or signs of disease present	once every spring and autumn	once every spring and autumn	once a year in not infected farm ever 2nd year in infected farm
N of fish sampled/ sampling time	1-10	30	30	30
Sampling from diseased fish	always	if noticed	if noticed	if noticed
N of pools for virus isolation/ sampling time	1-5	6	6	3
N of pools tested by qRT-PCR/ sampling time	1-5	6	6	3
N of serum samples/ sampling time	0	30	30	0
Temperature	<15 °C	<15 °C	<15 °C	<15 °C

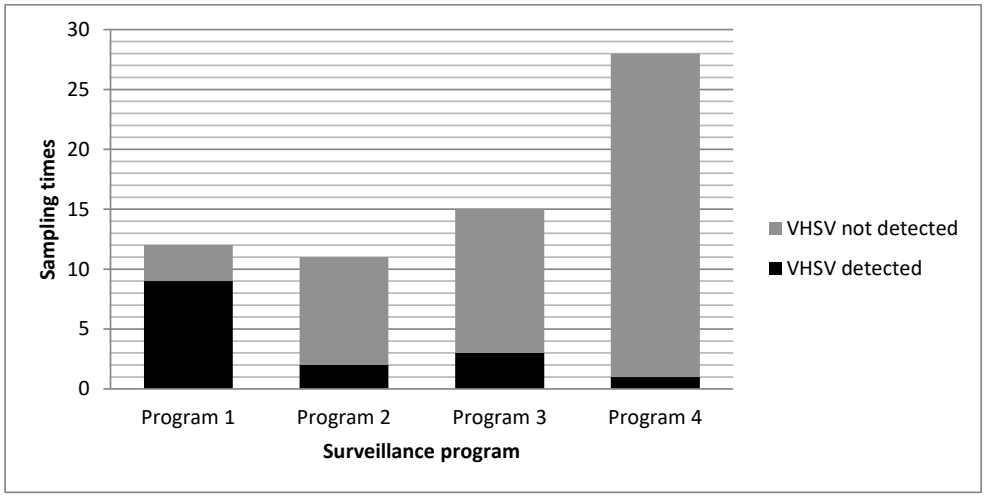

Figure 12 *Amount of sampling times in different surveillance programmes and how often VHSV was isolated from organ samples in cell culture.*

5.2 Wild fish and whitefish (II)

VHSV was not detected in any of the 1,636 wild fish, representing 17 different fish species, that were caught and tested for VHSV in the vicinity of infected fish farms. Four pooled samples of four fish species (ruffe *Gymnocephalus cernuus*, herring *Clupea harengus membras*, rainbow trout *Onchorynchuss myciss* and four horn sculpin *Triglopsis quadricornis*) gave a weak signal (Ct >36) with qRT-PCR (Table 3). These four samples tested negative with the strand-specific RT-PCRs, indicating that VHSV replication had not occurred in the tested samples.

The wild perch and roach that were exposed to VHSV at a fish farm with rainbow trout experiencing a clinical outbreak did not test positive during the infection trial. On the contrary, whitefish which were also exposed in the same way as perch and roach tested positive for VHSV. One of the three parallel groups of exposed whitefish was positive with both virus isolation and qRT-PCR. In addition, the strand-specific RT-PCR method, testing for positive-stranded RNA products that are present in a fish cell only during virus replication also gave a positive result. The isolated VHSV strains from the challenged whitefish and diseased rainbow trout of the fish farm, where the challenge was performed, and from other VHS -positive fish farms during 2001 and 2004 were sequenced and found to be almost identical; nucleotide identity was 99.4-99.9% (Figure 13). All the control fish in the control farm outside the restriction area tested negative for VHSV.
A Sprivivirus was isolated from all fish species in the challenge test. Spriviviruses grow in the same cell cultures as those used for VHSV isolation and were confirmed in a study performed by Holopainen et al. (2017).

Table 3 *Amount of wild fish tested for VHSV at farms with VHSV positive rainbow trout during 2005-2008. VHSV could not be isolated from these fish species. *Four species gave a weak positive (C(t)> 36) reaction, in one organ pool per species, by qRT-PCR: real-time reverse transcription PCR, but tested negative with the positive strand specific qRT-PCR.*

Species	Autumn	Spring	Sum.
Perch (*Perca fluviatilis*)	300	213	513
Bleak (*Alburnus alburnus*)	70	329	399
Roach (*Rutilus rutilus*)	168	97	265
Three-spine stickleback (*Gasterosteus aculeatus*)	2	151	153
Ruffe (*Gymnocephalus cernuus*)*		87	87
Herring (*Clupea harengusmembras* L.)*	30	46	76
White bream (*Abramis bjoerkna*)	30	36	66
Smelt (*Osmerus eperlanus*)		32	32
Rudd (*Scardinius erythrophthalmus*)	4	16	20
Rainbow trout (*Oncorhynchus mykiss*)*	1	6	7
Four horne scoulpin (*Triglopsis quadricornis*)*	2	4	6
Flounder (*Platichthys flesus*)	1	4	5
Sea trout (*Salmo trutta*)		2	2
Straight nosed pipefish *(Neropsis ophidion)*	2		2
Ealpout (*Zoarces viviparus*)		1	1
Ide (*Leuciscus idus*)	1		1
Pike (*Esox lucius*)		1	1
sum.	611	1025	1636

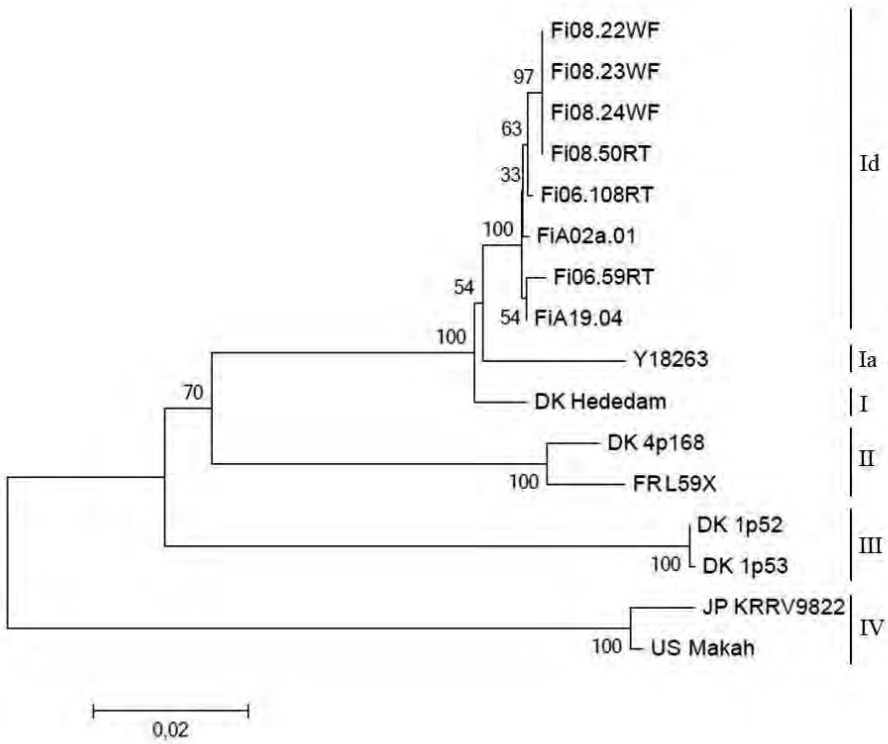

Figure 13 *Phylogenetic analysis of Finnish VHSV- strains isolated from white fish (Fi08.22WF, Fi08.23WF, Fi08.24WF) and rainbow trout (Fi08.50RT, Fi06.59RT, Fi06.108RT, FiA02a.01, FiA19.04) together with selected strains of VHSV belonging to genotypes I-IV. Tree is based on complete coding sequence of the glycoprotein (G) gene (1524 nt), and it was generated by using the neighbour-joining method in MEGA 4.1 software (Tamura et al. 2007). The reliability of the tree was determined by 1000 dataset bootstrap resampling; numbers on the tree represent percentage of bootstrap support. The scale bar indicates nucleotide substitutions per site. (Vennerström et al. 2018, Supplement)*

5.3 Blue mussels (III)

VHSV was not detected in any of the 62 pools of 193 wild blue mussels tested (CI 0.00-0.06), except for one pool that gave a weak signal Ct> 36 by qRT-PCR in May 2006 from samples from the infected fish farm. The results are presented in table 4.

According to the two bath challenge trials performed in this study, VHSV does not replicate in blue mussels exposed to live VHSV (Table 5).

In the first trial using virus suspension, VHSV was only isolated from the cell culture of the organ suspension of mussels from group I at the end of the 6 h exposure. All organ

suspensions of mussels from group II, exposed to the virus for 1 d, were negative by virus isolation (CI 0.05-0.23). qRT-PCR gave positive signals for VHSV RNA in both test groups I and II throughout the whole trial of 6 days.

In the second infection trial, blue mussels were exposed to VHSV by keeping them in an aquarium with VHSV-infected rainbow trout. The virus could not be isolated from the exposed blue mussels at any time of the trial (CI 0.00-0.07). VHSV RNA was detected in the organ suspensions of the mussels for 3 days, but weak signals (Ct>36) were detected throughout the whole 29-day trial. In the second trial, samples from the aquarium water were also collected and tested by virus isolation and qRT-PCR for VHSV RNA. VHSV could not be isolated from the aquarium water at any time, but VHSV RNA was detected in the aquarium water at the time when the rainbow trout were removed from the bath challenge.

Table 4. *Results of testing for viral haemorrhagic septicaemia virus (VHSV) in the hepatopancreas of wild blue mussels from two VHS-positive fish farms (A and B) farming rainbow trout for consumption in the Province of Åland and from a similar farm situated in a VHS-free zone on the west coast of continental Finland used as a control (C). N = number; Nd = not done; RT-PCR = reverse transcriptase polymerase chain reaction. [a] weak signal with threshold cycle cut-off >36 (Vennerström et al. 2020).*

			N pools positive / N pools tested	
Farm / Time of sampling	N mussels	N pools	Cell culture	Real-time RT-PCR
Company 1				
A / April 2006	13	7	0/7	Nd
A / May 2006	10	10	0/10	1[a]/10
A / November 2006	100	20	0/20	0/20
Company 2				
B / May 2006	10	5	0/5	Nd
B / June 2006	50	10	0/10	0/10
Control farm				
C / May 2007	10	10	0/10	0/10
Total	193	62	0/62	1[a]/50

Table 5. *Viral haemorrhagic septicaemia virus (VHSV) isolations and real-time reverse transcriptase polymerase chain reaction (RT-PCR) results from two bath challenges of blue mussels with VHSV grown in cell culture and VHSV from infected rainbow trout. In both trials, Groups I and II are test groups and group III is a negative control group of which all results were negative and are not shown in the table. d = days; h = hours; N = number; Nd = not done; RT-PCR = reverse transcriptase polymerase chain reaction. [a] weak signal with threshold cycle cut-off >36. (Vennerström et al. 2020)*

		N samples positive / N samples tested		
		Hepatopancreas samples of mussels		Aquarium water
Time of sampling	Group	Virus isolation in cell culture	Real-time RT-PCR	Real-time RT-PCR
Bath challenge with VHSV				
0 (before challenge)	I, II, III	0/5	Nd	Nd
6h (at end of challenge)	I	5/5	5/5	Nd
1d	I	0/5	3/5	Nd
1d (at end of challenge)	II	0/5	4/5	Nd
2d	I	0/5	1/5	Nd
2d	II	0/5	2/5	Nd
3d	I	0/5	0/5	Nd
3d	II	0/5	4/5	Nd
6d	I	0/5	3/5	Nd
6d	II	0/5	2/5	Nd
Bath challenge with VHSV-infected rainbow trout				
0 (before challenge)	I, II, III	0/3	0/3	0/1
At end of 10-min challenge	I	0/2	2/2	2/2
At end of 20-min challenge	II	0/2	1/2	2/2
1d	I	0/2	1/2	1[a]/1
	II	0/2	1[a]/2	1[a]/1
2d	I	0/2	2/2	1[a]/1
	II	0/2	0/2	1[a]/1
3d	I	0/2	1/2	0/1
	II	0/2	0/2	0/1
4d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
6d	I	0/2	1[a]/2	0/1
	II	0/2	1[a]/2	0/1
8d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
11d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
14d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
22d	I	0/2	1[a]/2	0/1
	II	0/2	0/2	0/1
27d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
29d	I	0/2	1[a]/2	0/1
	II	0/2	0/2	0/1

5.4 Sea water, wastewater and sediment (III)

The results of this study are presented in table 6. VHSV RNA was detected in one sample out of 40 filtered sea water samples collected in April–May 2008 from two fish farms with rainbow trout experiencing a clinical outbreak of VHS (CI 0.004-0.129). The positive sample was collected from the surface of a farm situated nearby a processing plant processing VHSV-positive rainbow trout. Virus isolation was not performed from these samples.

All four sea water samples collected in January and March 2009 were positive for VHSV RNA (CI 0.51-1.0). These samples were also filtered before testing by qRT-PCR. Virus isolations by cell culture performed from these samples were all negative.

All 10 sediment samples collected next to a net pen with clinically diseased rainbow trout were negative for VHSV RNA (CI 0.0-0.28).

All liquid waste samples collected from a processing plant processing VHSV-infected rainbow trout tested positive by qRT-PCR in January 2009 (CI 0.43-0.90). The positive samples were collected before the liquid waste was treated with disinfectants. VHSV was isolated by cell culture in 78% of the same samples. After the final disinfection of the liquid waste, no virus could be detected by either method.

In March 2009 the sampling from the processing plant was repeated, but only clinically healthy whitefish were processed at the processing plant. VHSV was isolated from 63% of the samples (CI 0.31-0.86). The liquid waste disinfection system was not running at the time of the second sampling, and therefore disinfected effluent could not be collected for testing.

Table 6. *Results of testing for viral haemorrhagic septicaemia virus (VHSV) in sea water, sediment and liquid wastewater from two VHSV-positive fish farms and a plant processing VHSV-positive fish. CPE = cytopathic effect; N = number; Nd = not done; pos = VHS-positive samples; PP = processing plant of company 2; RT-PCR = reverse transcriptase polymerase chain reaction; x = water and liquid waste samples were filtered before testing with real-time RT-PCR (Vennerström et al. 2020).*

				Virus isolation	Real-time RT-PCR	
Farm / time of sampling / water temperature	Type of sample / Origin of sample	N samples (pooled for direct real-time RT-PCR)	Water filtering	N CPE pos / N samples	N pos / N CPE pos cell culture	N water samples pos / N tested
Company 1						
A / April–May 2008 / 4°C	Seawater / net pens with VHSV - positive trout	21	x	Nd	Nd	1/21
A / April 2008 / 4°C	Sediment / under net pens with VHS-positive trout	10		Nd	Nd	0/10
Company 2						
B / May 2008 / 10°C	Seawater / net pens with VHS-positive trout	19	x	Nd	Nd	0/19
PP / January 2009 / 2°C	Seawater / net pens with VHS-positive trout	3(1)	x	0/3	Nd	1/1
	Seawater / loading dock of slaughterhouse	3(1)	x	0/3	Nd	1/1
PP / January 2009 / 2°C	Liquid waste / stunning basin	3		2/3	2/2	3/3
	Liquid waste / bleeding basin	3		1/3	1/1	3/3
	Liquid waste / kidney remover	2		2/2	2/2	2/2
	Liquid waste / drain before disinfecting	3		3/3	3/3	3/3
	Liquid waste / drain after disinfecting	3		0/3	Nd	0/3
PP / March 2009 / 0°C	Seawater / loading dock of slaughterhouse	2	x	0/2	Nd	2/2
PP / March 2009 / 0°C	Liquid waste / stunning basin	2(1)		1/2	1/1	1/1
	Liquid waste / bleeding basin	2(1)		2/2	2/2	0/1
	Liquid waste / kidney remover	2(1)		2/2	2/2	1/1
	Liquid waste / drain before disinfecting	2(1)		0/2	Nd	0/1
	Liquid waste / drain after disinfecting	Nd	Nd	Nd	Nd	Nd

6. Discussion

This thesis consists of several observational studies conducted at fish farms and processing plants, farming or handling VHSV-infected rainbow trout or whitefish during the study period. Also, several experimental field and laboratory challenge tests were performed. The farms were situated in a restriction area regarding VHS since 2000 with strict restriction concerning transport of live fish and farming equipment out from this area. Within the restriction area, fish farming continued despite the presence of VHS, and processed fish could be transported out from the restriction area. In the restriction area, the infrastructure of the fish farming activity did not take into account the possible spread of fish diseases. Movement of fish was conducted in a way whereby different age classes met several times and connection to processing plants was continuous. Efforts were however made to sanitise fish farms regarding VHS, mainly the summer farming localities that were emptied of fish every autumn. Winter farming localities were usually never completely emptied of fish and sanitised. This was typical especially for farm A, which was situated next to a processing plant and fish were continuously moved between winter locality and the nearby summer locality. The movement of fish at farm B was mostly in one direction, from fry farming to processing plants, but daily contacts from the processing plants to most of the farming localities existed due to the daily servicing of the farms. When this study was planned, it was quite clear that VHS would be almost endemic at the fish farms in the study area, which gave an excellent opportunity to study different ways of screening for the disease and factors that could affect disease eradication. In the following sections all the objectives of the study are discussed.

6.1 Surveillance of VHSV infection in rainbow trout populations (I)

In 2006, the European Commission released a new directive for its member states concerning health requirements for aquaculture and prevention and control of certain diseases including VHS (EC 2006). The disease surveillance was to be risk-based in all aquaculture areas, a demand that was not easy to implement in countries where the fish farming industry is small-scale, and no fish health services are available. Risk-based approaches specially directed to these aquatic animal diseases were not presented in this directive or other resolutions of EC at that time (Oidtmann et al. 2013). One important aspect of the risk-based surveillance is the demand that samples should always be sent in for diagnostics if any suspicion of fish disease is raised. In Finland, laboratory services, for diagnosing fish diseases have been available for decades, but there have been no fish health veterinarians working in the field to help farmers with practical health problems. Especially in food fish farming it was not a common custom to contact a veterinarian due to health issues, and samples were usually sent only during summer when bacterial diseases cause high mortalities. The reason for the lack of fish health services is that the Finnish fish farming industry is small and scattered over a large area, making private fish health services

unprofitable. In the study area, sampling for viral fish diseases was mainly conducted during official inspections, and the official screening programme was not risk-based but solely active surveillance. Active surveillance is based on the competent authority's own activity to secure sampling and reporting according to legislation.

6.1.1 Surveillance procedures

In the first study (I), one of the surveillance programmes (programme 1) was conducted by the farmers (farms A and B), who were promised free diagnostic services if they agreed to send in samples every time they noticed signs that could indicate fish disease in their fish populations. Previously, farmers only sent samples when high mortalities were seen; mild signs of disease were often not reported or confirmed. VHSV Id was reported to cause 40% mortalities in an infection trial (Raja-Halli et al. 2006), but such high mortalities were rarely reported in the study area (author's unpublished data). Mortalities ranging from 10% up to 50% have only been detected after stressful events, such as the transportation of infected populations between farming localities or to processing plants.

This syndromic surveillance was the most reliable means of screening for the presence of VHSV, being up to 17 times more effective than the active surveillance in programme 4 carried out by the competent authority. This result indicates that active surveillance only using methods to detect viruses, although regarded as sufficient to show infection in an area, is not a reliable tool to reveal whether a single population is or has been infected by VHSV. It is notable that Programme 3, with sampling after a clinical VHS outbreak, performed almost as well as Programme 1, while the other two sampling programmes (2 and 4) performed less efficiently. In practice, an active surveillance procedure (programme 3) that is performed after a confirmed clinical VHS outbreak would not be a sensible strategy to identify VHSV-infected farms, as they have already been found. In contrast, syndromic surveillance outperformed active surveillance programmes and has clear practical value.

The farmers that carried out the syndromic surveillance in programme 1 had six years' experience of VHS outbreaks and good skills in detecting abnormalities indicating a disease outbreak in the early stages of infection in their fish populations. This experience is considered an important factor in the good result of programme 1. The finding that temperature affected the performance of the programmes might be associated with this: fish farmers found the occurrence of clinical cases at certain temperatures to be typical of the disease. The staff of the study fish farms were also motivated to participate in this surveillance, and new means of transportation of samples to the laboratory were found, which was also vitally important for this field study. In programmes 2 and 3, the populations were also carefully observed at the time of sampling, but only the farmers were able to follow up their fish populations daily. Sampling implemented in the early stages of the infection when clinical symptoms can be observed have a higher possibility of finding test positives, as there are high quantities of the virus present. Sampling performed as part of active surveillance is mainly successful for testing carriers of disease with low or undetectable amounts of the disease agent.

The water temperature in the study area varies from slightly above 0°C in winter to often above 20°C in summer. In autumn, the water temperature drops below 15°C in late September, and ice may cover the farming localities from December to late April. This ice layer can make inspection and sampling impossible for several months. When the ice layer melts in spring, the water temperature often rises above 15°C within 2–3 months, varying from year to year. This gives a short time window for the authorities to visit the farms, which are scattered around thousands of small, difficult to reach islands. The fish populations are therefore often sampled at the processing plant, where disease signs are difficult to notice.

Sandlund et al. (2014) reported that gills are useful target organs in screening chronic or sub-acute VHSV infections. Therefore, it could be argued that programme 1 would not differ as much from the other programmes if the gills had also been tested in the other programmes. However, gills were used on two occasions in parallel with the other organ samples of the same fish in this study without gaining any new information (Vennerström, unpublished data).

6.1.2 Diagnostic methods used for screening

Both virus isolation in cell culture and real-time RT-PCR are reliable tests for detecting VHSV when there is an acute VHSV infection at the time of sampling, but effective early warning systems are required to detect signs of the disease. Real-time RT-PCR is a rapid and reliable test to confirm or rule out the presence of VHSV in organ suspension when clinical signs have given reason to suspect infection. Real-time RT-PCR is also reported to be valuable in finding asymptomatic fish carrying VHSV (Hope et al. 2010). In our study, real-time RT-PCR detected a possible carrier on one occasion when virus isolation in cell culture failed. This was a situation in spring 2007 when the water temperature was close to 15°C and rising. It is possible that this population had just been infected and clinical signs of VHS had not appeared before the water temperature rose above 15°C. We have noticed that outbreaks due to VHSV Id do not occur and the virus cannot be isolated at temperatures higher than 15°C. Serum samples that were collected on the same occasion did not reveal any antibodies against VHSV, also suggesting an early infection. Our suspicion concerning the carrier state was confirmed the next autumn, when the water temperature dropped below 15°C and the fish in this population experienced a clinical disease outbreak. Serum samples taken at this time revealed only one positive sample out of 15 tested. This suggested a new infection and indicated that the virus infection did not have time to spread in the population before the water temperature rose above 15°C during the previous spring.

Real-time RT-PCR is valuable when screening for a particular virus, e.g. in wild fish, and where a positive signal does not lead to legal actions against the business owner. Real-time RT-PCR could also be used as the primary diagnostic screening test for fish farms, but a positive result should be confirmed using other methods such as sequencing of positive products and molecular epidemiology. Use of serological testing of antibodies against VHSV from fish serum is also possible.

Screening for antibodies against VHSV using ELISA and PNT has been reported as useful methods in surveillance of VHSV-infected populations (Fregeneda-Grandes and

Olesen 2007, Fregeneda-Grandes et al. 2009, Schyth et al. 2012, Millard et al. 2014, Wilson et al. 2014). In our study, we tested fish sampled in programmes 2 and 3 for antibodies against VHSV using an ELISA method. We detected antibodies against VHSV several months after a clinical disease outbreak had occurred. However, the results were easy to interpret only if there had been a clear clinical outbreak no more than one year earlier in the population.

Early detection of VHS is essential for successful disease eradication. Virus infections are easily spread between farms in the same area due to daily management practices. Routine clinical inspections performed by skilled fish health specialists have also been noted as essential in the surveillance of freedom from VHS in Norwegian marine salmon farms (Lyngstad et al. 2016). Our study supports the Norwegian report by demonstrating that more frequent monitoring for clinical signs of VHS outperforms active surveillance. There are no fish health services offering routine clinical inspection or sampling services for the fish farms in the study area. The farming localities are difficult to reach and shipping of samples by the farmers themselves for testing of diseases is complicated, as the logistics involved in transferring samples between the study area and the laboratory are often poor.

The successful eradication of VHSV in two other areas on the south coast of Finland in 2001 and 2003 (reinfection 2008) could be explained by the early detection and rapid eradication of the affected farming localities, which is vital for the eradication of and subsequent freedom from disease. Farmers contacted the authorities immediately when they noticed suspicious disease signs. Eradication was performed without delay and in good cooperation between farmers and authorities. In Åland, this cooperation was not as successful at the beginning, VHSV rapidly spread between farming localities and stamping out the disease was not economically justified. We believe that this study managed to improve the screening of VHSV and biosecurity measures in this area. According to the official disease surveillance in the restriction area in Åland, the number of VHS-positive samplings has followed a decreasing trend (ICES 2014), which indicates a lower infection pressure in the area. VHSV has not been isolated in the study area or anywhere in the Åland Islands since 2012.

6.2 Studies on wild fish and farmed whitefish (II)

6.2.1 Wild fish

Wild fish have been reported to be carriers of several different VHSV genotypes in the North Sea and the Baltic Sea (Skall et al. 2005ab). However, there are no reports indicating that wild fish would relate to the clinical disease caused by VHSV genotype Id in farmed fish. The number of different fish species in the close vicinity of the fish farms in this study is high, but the number of individuals of each fish species varies from only a few individuals like pike or sea trout to thousands like perch, roach or three-spined stickleback. In our study, the observed prevalence of VHSV was zero in 17 different tested wild fish species that were

screened during several years in the vicinity of two infected fish farms. Additionally, wild perch and roach did not become infected when they were challenged with VHSV by keeping them in small cages close to rainbow trout experiencing a clinical outbreak of VHSV.

Wild fish were not found to be a likely source for the reappearing VHS outbreaks in the Finnish brackish water fish farms. The results indicated that if the screening had missed the infection in species caught in quite high numbers the prevalence would have been no more than 4%. The sample size was low for defining the possible prevalence range of some species and the results are inconclusive for them. These fish species that were caught in small numbers comprise less than 5% of all fish analysed. The species in question have a low apparent prevalence on the farms and in their vicinity. In addition, a fish farm is not a normal habitat for these fish species and even a small number of these species probably represent a sufficient portion of the individuals present. Organ pools from fish species caught in low numbers contained less individuals than species caught in high numbers, reducing the possible effect of virus dilution. It is possible that some of the tested fish species could be transient carriers of the virus and therefore not caught by the screening method used. More studies on the prevalence of VHS in wild fish are needed, as all fish catchment methods have species and size selectivity that may induce bias in the results.

The minor role of the wild fish being the source for reappearing VHS outbreaks in farmed rainbow trout in the study area is supported by several facts. The farms where the wild fish were caught experienced several outbreaks of VHS in their fish during the study. If the wild fish had a major role, one would expect to find clear positive signals with real-time RT-PCR from the tested wild fish, as VHSV was present in the environment on several of the sampling occasions. Another fact indicating the minor role of wild fish is that VHSV was successfully eradicated at the first attempt from the other two restriction areas regarding VHSV in Finland with similar farms producing rainbow trout and the same kind of wild fish populations as the farms in the study area in the Province of Åland.

Further support for the minor role of wild fish is that extensive screening of wild herring and sprat has been conducted on the coast of Finland without finding any samples positive for VHSV Id (Gadd et al. 2010). It was believed that herring on their spawning migration would have brought the infection to Finnish fish farms in the same manner as was reported on the west coast of Sweden in 2000 (Nordblom and Norell 2000). In Sweden, the same type of VHSV Ib was isolated from both herring caught close to infected rainbow trout farms and from the rainbow trout on these farms. It was assumed that the herring on their spawning migration brought the infection repeatedly to the farm and eventually made rainbow trout farming in that area impossible (Nordblom and Norell 2000, Jansson and Vennerström 2014). This has not been the case in Finland, and there is no indication that eradication measures should be omitted because of wild fish.

6.2.2 Farmed whitefish

In our study, farmed whitefish, challenged in the same way as perch and roach, were infected by VHSV Id and the virus replicated in the tested organs. The virus could be detected for a short period, but no mortalities were recorded. Our results are supported by

the report from Skall et al. (2004a) of an infection trial with farmed whitefish where they found whitefish to suffer only low mortality after infection with a VHSV Ib strain isolated from marine fish. They also found that infected fish continued to carry the virus for at least the three weeks that the trial lasted. VHSV has also been reported in whitefish in Switzerland and Germany (Ahne and Thomsen 1985, Meier et al. 1986).

Whitefish had become a quite common farmed food fish species in the study area a few years before VHSV was detected for the first time in Åland in 2000. Whitefish was often farmed on same farms as rainbow trout, but according to the Finnish authorities, VHSV has only once been isolated from farmed whitefish in the restriction area regarding VHSV (Vennerström, unpublished data). Whitefish should be considered potential carriers of VHSV and a source of the recurring VHS outbreaks in the VHS-restriction area of Åland. Whitefish is a native species of the Baltic Sea, where VHSV is endemic. According to phylogenetic studies undertaken by Einer-Jensen at al. (2004) the Finnish rainbow trout isolates (VHSV Id) are closest to the common ancestor of marine (VHSV Ib) and freshwater isolates. Perhaps whitefish that is a native fish species of the Baltic Sea has developed a genetic resistance to marine strains of VHSV and is therefore not as sensitive to the disease as the imported non-native rainbow trout. This is supported by the fact that mortality caused by VHSV Id in Finnish whitefish has never been reported, although whitefish are also screened for VHSV repeatedly according to regulations set up by the competent authority.

Since whitefish may be infected by VHSV Id and the virus can replicate in this fish species, whitefish kept close to VHSV-positive rainbow trout populations may give the virus an opportunity to 'jump' to whitefish and survive longer in the area. This may also give the virus an opportunity to adapt and become more virulent to whitefish if given the opportunity to jump between species by farming several fish species on the same farms or close to each other. This may have been the case in Norway, where VHSV of genotype III was isolated from farmed rainbow trout on the west coast in 2007. Genotype III could be considered endemic among wild fish e.g. cod in the North Sea (Snow et al. 2000, King et al. 2001, Smail 2000). According to earlier infection trials, rainbow trout has not been sensitive to this genotype (Skall et al. 2004b). In 2007, a new type of VHSV genotype III was isolated from rainbow trout that was pathogenic to rainbow trout (Dale et al. 2009). The source of the infection is not clear, but these positive farms had close connections to cod (*Gadus morhua*) and saithe (*Pollachius virens*) farming where raw processed fish of marine origin were used for feed. Containers of dead farmed rainbow trout, cod and saithe from other localities were stored close to the primary infected locality (Dale et al. 2009).

It could also be possible that whitefish can be transient carriers of VHSV, as the virus was not detected in the whitefish groups after the infected rainbow trout were mowed away from the farm.

6.2.3 Sprivivirus

Sprivivirus was isolated from all tested fish species (perch, roach and whitefish) in the infection trial, but was not associated with mortalities. These findings were not studied further in this trial. Sprivivirus has occasionally been isolated from farmed sea trout in the

study area in connection with bacterial fish diseases during the summer when water temperatures are over 15°C (personal communication Holopainen R.). Sprivivirus seems to be an endemic virus in the study area and it is not clear whether this virus could be a predisposing factor for the VHSV outbreaks or influence the screening of VHSV. Sprivivirus has not been reported from the two successfully eradicated restriction areas of VHS in Finland.

6.3 VHSV in the environment of fish farms and processing plants (III)

6.3.1 Blue mussels

Based on the results from our studies on blue mussels, it can be assumed that the VHSV is not able to replicate in blue mussels and is quickly inactivated in them. This was shown by taking samples from the hepatopancreas of mussels living in VHSV-infected fish farms and by two different infection trials using high doses of VHSV. The challenges were performed using two different methods, but the result was the same regardless of the method used.
The rapid inactivation of VHSV in sea water, but somewhat longer persistence of VHSV RNA in mussels, was observed in our study (especially in the second challenge test). Blue mussels may serve as a physical attachment surface for VHSV. Thus, it is possible that mussels can protect VHSV in sea water from environmental effects that could destroy the virus and may prolong the viral contamination of the environment even if the fish farms are fallowed. The difference could be a result of the frequent water changes in the test aquariums that were performed to give the mussels as good conditions in the aquarium as possible. VHSV is an enveloped virus that is not as resistant to environmental effects as birnaviruses, which have no envelope and have been found in free-living molluscs (Mortensen et al. 1992, Rivas et al. 1993, Bovo et al. 2005). However, the replication of VHSV in mussels was unlikely, otherwise increased secretion of the virus would have occurred in the mussels and one would have expected the virus load in the aquarium water to increase as well. The result is also indirectly supported by the fact that VHS was successfully eradicated in two similar farming localities farming rainbow trout for consumption on the west and south coast of Finland (Raja-Halli et al. 2006). These farms also had high densities of blue mussels in their environment. If VHSV could replicate in mussel tissues, one would expect those eradications to have failed. Similar results have been shown in studies with infectious salmon anaemia virus ISAV (Skår and Mårtensen 2007).

6.3.2 Liquid waste and sediment

VHS virus was more frequently detected in sea water close to the net pens with VHSV-diseased rainbow trout populations and in liquid waste from processing plants handling

VHSV-positive fish in cold water temperatures during winter than in spring. The water temperature in the study area was close to 0°C in January–March and 4–10°C in April–May. Daylight is only 6 h in January but increases to 14–16 h in April–May (Nordlund 2017, Cornwall et al. 2018). The low amount of UV radiation in winter (Finnish meteorological institute 2019) in the study area together with the short daylight hours and cold-water temperature could explain the difference in virus survival. The result is consistent with previous studies where VHSV was reported to be sensitive to UV light and to survive longer in cold water temperatures than in warm (Ahne 1982, Parry and Dixon 1997, Øye and Rimstad 2001, Yoshimizu et al. 2005, Hawley and Garver 2008).

Liquid waste samples from the processing plant collected in March 2009 were positive for VHSV RNA, although only clinically healthy whitefish had been processed at the time. Although these whitefish were not sampled in this study, we have noticed in a previous study that although whitefish are not easily infected with VHSV genotype Id, some fish in the population may become infected and virus replication occur (Vennerström et al. 2018). The processed whitefish were farmed next to the processing plant where VHSV-positive rainbow trout had been processed earlier the same year, and it is possible that the virus was transmitted. Another possibility for this virus-positive finding is that the processing line was highly contaminated by VHSV RNA from infected rainbow trout processed earlier. It could well be assumed that processing plants handling VHSV-positive fish and the surrounding environment are heavily contaminated with the virus, especially in winter. For this reason, any contact between these plants and susceptible farmed fish populations should be avoided, especially during the coldest and darkest time of the year.

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7. Conclusion

Syndromic surveillance (based on the observation of clinical disease signs in fish by fish farmers) is more sensitive than active surveillance when detecting VHSV infections on fish farms. Active surveillance (programmes 2, 3 and 4) did not yield information that would have been needed for preparing eradication plans in the VHS-eradication area of Åland.

Real-time RT-PCR was at least as reliable as virus isolation in cell culture to detect infection in this study. Serology proved to be a useful test to determine whether a fish population had been infected with VHSV. However, the antibody levels are very low in mild disease outbreaks or if the infection has occurred several months earlier. Therefore, the use of this antibody test in screening for VHSV in disease-free areas is not reliable without affirmation of the test results using another test, such as virus isolation or PCR. On the other hand, it is a useful additional tool in VHSV eradication for screening populations during the follow-up period, before declaring an area free of infection.

Processing plants handling VHSV-positive fish and sea water close to VHSV-positive fish populations are contaminated with VHSV especially during winter when daylight is sparse, and water temperatures are close to zero. Contact with contaminated localities increases the risk of the disease spreading to susceptible fish populations. Based on our results, blue mussels are not a relevant source of VHSV, as the virus is rapidly inactivated in mussel tissues, but they could provide VHSV with a physical protective environment that could prolong the survival time of the virus, although probably not for more than a few days.

According to our study, wild fish living freely in the fish farming area do not seem to threaten the farmed fish with respect to VHSV genotype Id infection in Finland. Farmed whitefish as a native species was a possible source of the recurring VHS outbreaks in Finnish brackish water food fish farms, as they were infected but seemed to clear out the infection. Wild fish may function as carriers of virus between closely situated farms. Therefore, it is important to perform quick stamping-out of infected fish farms and decrease the infection pressure and adaptation possibilities in other fish species.

Early detection of VHSV infection is crucial for VHS management. The personnel working on fish farms have the key role in disease management as they are the only persons that can notice even small changes in their fish populations, indicating a possible infectious disease. In addition, it is important to have a good cooperation between farmers and the fish health specialist so that even small suspicions would lead to testing for possible infections.

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9 Original publications

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Viral haemorrhagic septicaemia virus (VHSV Id) infections are detected more consistently using syndromic vs. active surveillance

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ABSTRACT: The eradication of viral haemorrhagic septicaemia virus (VHSV Id) from Finnish brackish-water rainbow trout *Oncorhynchus mykiss* farms located in the restriction zone in the Province of Åland, Baltic Sea, failed several times in the 2000s. The official surveillance programme was often unable to find VHSV-positive populations, leading to the misbelief in the fish farming industry that virus eradication could be achieved. The ability of 3 other surveillance programmes to detect infected fish populations was compared with the official programme. One programme involved syndromic surveillance based on the observation of clinical disease signs by fish farmers, while 2 programmes comprised active surveillance similar to the official programme, but included increased sampling frequencies and 2 additional tests. The syndromic surveillance concentrated on sending in samples for analysis when any sign of a possible infectious disease at water temperatures below 15°C was noticed. This programme clearly outperformed active surveillance. A real-time reverse transcriptase-polymerase chain reaction method proved to be at least as sensitive as virus isolation in cell culture in detecting acute VHSV infections. An ELISA method was used to test fish serum for antibodies against VHSV. The ELISA method may be a useful tool in VHSV eradication for screening populations during the follow-up period, before declaring an area free of infection.

KEY WORDS: Rainbow trout · *Oncorhynchus mykiss* · *Novirhabdovirus* · *Rhabdoviridae* · Virus eradication · Fish farm · Aquaculture · RT-PCR · ELISA · Finland

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a fish disease that has been described in more than 80 fish species in both fresh and marine waters (review by Skall et al. 2005a, Elsayed et al. 2006, Lumsden et al. 2007, Dale et al. 2009, Bain et al. 2010, Gadd et al. 2010, 2011, Kim & Faisal 2010, Emmenegger et al. 2013, Ito & Olesen 2013). The disease is caused by VHS virus (VHSV), a virus belonging to the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Walker et al. 2000). VHSV is a single-stranded enveloped RNA virus that is divided into 4 genotypes (I–IV), of which I and IV have several sublineages (Ia–Ie, IVa–IVc) (Snow et al. 1999, Einer-Jensen et al. 2005, Elsayed et al. 2006, Ammayappan & Vakharia 2009, Pierce & Stepien 2012). The susceptibility to different VHSV genotypes varies among fish species (Skall et al. 2005b, Schönherz et al. 2013). In aquaculture, VHS is a severe infectious disease of farmed rainbow

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trout *Oncorhynchus mykiss*, turbot *Scophthalmus maximus* and Japanese flounder *Paralichthys olivaceus* (Ross et al. 1995, Smail 1999, Isshiki et al. 2001). Infections caused by VHSV genotype I are among the most serious viral diseases in rainbow trout farming, causing mortalities of up to 80–100% in rainbow trout fry and 10–50% in fingerlings and older fish (Smail 1999). Due to its ability to cause severe disease in wild and cultured fish, VHSV is a notifiable disease to the World Organisation of Animal Health (OIE 2017). In North America, high mortalities have also been reported in several wild fish species infected with VHSV genotype IV (Meyers et al. 1999, Hedrick et al. 2003, Groocock et al. 2007, Lumsden et al. 2007, Garver et al. 2013).

In Finland, VHS was diagnosed for the first time in spring 2000 at a fish farm producing rainbow trout for consumption in open net pens in the sea area of the Province of Åland (hereafter called Åland; Fig. 1), and almost at the same time at a similar fish farm on the south coast of continental Finland approximately 330 km away (Raja-Halli et al. 2006). Infection with VHSV genotype Id spread rapidly between fish farms in Åland, despite extensive eradicative measures, and in 2001, a restriction zone including the whole province was established. Movement of live fish, ungutted farmed fish and fish farming equipment including well boats from the restriction area was forbidden. However, eradicative measures were successful in the second area on the south coast, and VHSV has not been isolated there since 2001. In 2003, VHSV spread from Åland to a third area, a fish farm also producing rainbow trout on the west coast of continental Finland (Raja-Halli et al. 2006). Infection was successfully eradicated in the same year and VHSV was not reported until 2008, when the virus was isolated again. Eradication was repeated and no infection has been recorded since 2008.

The disease situation in the restriction zone of Åland was screened according to official EU protocols and tests (2001/183/EC and 2003/634/EC; EC 2001, 2003). New disease outbreaks were often reported 1–2 wk after fish from a VHS-free area were moved to sites that had been empty of fish for 8–12 mo, including the removal and disinfection of all farming equipment (later fallowed).

The fish farming industry in Åland started to improve biosecurity on farms, but willingness to change the infrastructure to a higher biosecurity standard was low, as wild fish were believed to be the source of reinfections. Initial phylogenetic reports of the Finnish farm isolates hypothesized that wild fish populations were the source of the primary infection (Raja-Halli et al. 2006). Nevertheless, surveillance efforts that screened wild herring, sprat, salmonid brood fish and lampreys *Lampetra fluviatilis* for VHSV between 2004 and 2006 on the west coast of Finland in the Baltic Sea, remained negative for VHSV Id (Gadd et al. 2010, 2011). Furthermore, subsequent screening of wild fish in the vicinity of the study farms reported on herein also suggested a lack of VHSV in wild fish (P. Vennerstöm unpubl. data). Recurrent outbreaks of VHS in Åland were difficult to explain, and suspicion about the surveillance programme was raised, including the diagnostic sensitivity in screening for the presence of VHSV infection. It was suspected that the surveillance programme and methods used were only able to find the 'tip of the iceberg' and that in order to achieve successful eradication, surveillance activities needed to be improved.

Here, we report the first part of a set of epidemiologic studies performed during 2006–2009 in the VHS restriction area of Åland, where VHSV was suspected to be present. More information on the presence of infection was needed to plan eradication measures. The aim was to compare 4 sampling strategies, later called programmes, to detect VHSV-infected fish in fish populations reared by 2 fish farming companies. Two diagnostic tests, a real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for

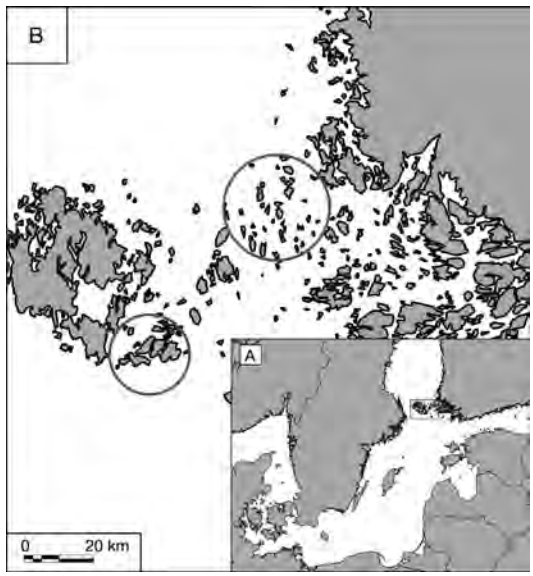

Fig. 1. (A) Geographical location of the Province of Åland (red rectangle), which is the viral haemorrhagic septicaemia restriction zone. (B) Study areas (circles) within the restriction zone

detecting viral nucleic acid from organ samples and an ELISA test for the presence of antibodies against VHSV in fish serum, were compared with the official EU tests, namely virus isolation by cell culture combined with an antigen detection ELISA, which is considered the reference method (OIE 2017). Another aim was to test whether syndromic surveillance by the staff on the farms would provide enough information to plan the eradication of VHS.

MATERIALS AND METHODS

Description of fish farms and populations selected for surveillance and the study area

Two companies (A and B) from a VHS restriction zone in Åland (Fig. 1) that used open net pens at sea to produce rainbow trout for consumption were screened for the presence of VHSV in their fish populations. In this study, a population included fish of the same origin and age, even if they were farmed at the same site with fish of other origins and ages. Company A had 2 farming sites, A1 and A2. A1 was constantly populated, but site A2 was only used during summer when the water temperatures had risen. A2 was situated further offshore than A1 and was populated with fish intended to be slaughtered in the following autumn or winter. Company A constantly had several fish populations present (AP1, 2, 3, …), and the number of populations site^{-1} depended on the time of year. Sites A1 and A2 were considered as 1 epidemiological unit, as they were situated <1 km apart, had the same staff taking care of daily services, and the populations were circulated between these 2 sites. Company B had 15 farming sites, most of which were summer farming sites that were followed during the winter. These farming sites were further apart from each other than those in Company A, but were also populated with several populations circulating between different sites. The fish of both companies were transported from a VHS-free zone in continental Finland. Company A imported some of its populations from abroad from a VHS-free zone in Denmark (author's unpubl. data). In addition, the domestic fish populations in the study were examined by virus isolation in cell culture and found negative for the presence of VHSV before transportation to the selected farms. The foreign fish were sampled before they were placed in the net pens. In our experience, VHSV Id cannot be isolated at water temperatures >15°C. In the study area, this temperature was measured from late June to September–October in 2006–2008. All farm visits and sampling were performed when the water temperature was ≤15°C, except for 1 case in Programme 2, when the population arrived at the farm in June 2006 (see Tables 1 & 2). From December–January to early May, the water temperature was <5°C (in total, 18–24 wk annually) (Lehtiniemi & Lehtinen 2016). The salinity in the study area was ca. 5.4–6.0‰.

Surveillance programmes, times and sampling

All populations (P) in Companies A and B at a total of 17 separate farming sites (AP1–2, BP1–BP15) were observed by 4 simultaneous surveillance programmes, 1–4, during 3 yr, 2006–2008. The sampling schedule, collected samples, sites from where the populations were sampled and the tests used are summarized in Table 1 and described in more detail below and in Table 2.

Programme 1 was conducted from May 2006 to the end of 2008 and consisted of syndromic surveillance performed by the staff of the fish farms. As soon as any signs of a possible disease outbreak were detected, the staff, consisting of several persons, had been instructed to send 5–10 euthanized whole fish to the laboratory for autopsy and sampling. VHS outbreaks in Finland have not been reported at water temperatures above 15°C, and only samples taken

Table 1. Code of action in different surveillance programmes. Water temperature was <15°C on all sampling occasions

Programme		Sampling frequency		Number per sampling occasion			
No.	Description	Routine	Diseased fish	Fish sampled	Pooled samples: Virus isolation	Pooled samples: qRT-PCR	Serum samples
1	Syndromic	Always if mortality elevated or signs of disease present	Always	1–10	1–5	1–5	0
2	Active, random	Once every spring and autumn	If noticed	30	6	6	30
3	Active, non-random	Once every spring and autumn	If noticed	30	6	6	30
4	EU reference	Once a year in uninfected farms; every 2nd year in infected farms	If noticed	30	3	3	0

Table 2. Results of surveillance for viral haemorrhagic septicaemia virus (VHSV) of fish populations during 2006–2008. In Programme 1, farmers from companies A and B performed syndromic surveillance and collected the tested samples. In the active surveillance of Programme 2, fish populations were randomly selected from Companies A and B. In the active surveillance of Programme 3, VHS-positive fish populations were non-randomly selected during 2007 and 2008 from Company B. In Programme 4, official EU surveillance of farms A and B was conducted by the competent authority according to Commission Decisions 2001/183/EC and 2003/634/EC (EC 2001, 2003). N: number; A: Company A; B: Company B; the number indicates the farming site of the company; P refers to population and the numbers indicate different populations; nd: not done and not included in statistical comparison of virus isolation and real-time (q)RT-PCR tests; na: not applicable to that programme; *A.s.*: *Aeromonas salmonicida* sp. *salmonicida* bacterial infection; PKD: proliferative kidney disease; RTFS: rainbow trout fry syndrome; *Y.r.*: *Yersinia ruckeri* bacterial infection; IPN: infectious pancreas necrosis; –: no information

Sampled sites	Population ID	Sampling date	Temp. (°C)	Clinical signs	N fish sampled (N organ pools)	N positive organ pools Virus isolation	qRT-PCR	N seropositive fish ELISA+ (N tested fish)	Diagnosis
Programme 1									
A1	AP1	May 2006	11	Yes	9 (9)	9	nd	na	VHS
A1	AP2	Sep 2006	15	Yes	4 (4)	0	0	na	*A.s.*, PKD
A1	AP2	Apr 2007	3	Yes	6 (4)	0	0	na	RTFS
A1	AP4	Apr 2008	4	Yes	3 (3)	3	3	na	VHS
A1	AP4	May 2008	7	Yes	4 (4)	4	4	na	VHS
A1	AP4	May 2008	7	Yes	4 (4)	4	4	na	VHS
A1	AP5	Sep 2007	13	Yes	14 (2)	0	0	na	*Y.r.*
A1	AP5	Mar 2008	2	Yes	5 (5)	5	5	na	VHS
B5	BP1	Jun 2006	12	Yes	10 (2)	2	nd	na	VHS
B6	BP6	May 2008	10	Yes	1 (1)	1	nd	na	VHS
B4	BP7	Jun 2007	12	Yes	10 (2)	2	2	na	VHS, RTFS
B4	BP7	Nov 2007	6	Yes	2 (2)	2	2	na	VHS
Total				12	72 (42)	32	20		
Programme 2									
A1	AP2	Jun 2006	18	No	60 (6)	0	0	nd	IPN
A1	AP2	Jan 2007	4	No	30 (6)	0	0	0 (15)	Negative
A1	AP2	Apr 2007	7	No	30 (6)	0	0	0 (15)	Negative
A1	AP2	Jun 2007	13	Yes	30 (6)	0	0	nd	*Y.r.*
A1	AP2	Nov 2007	7	No	30 (6)	0	0	0 (15)	Negative
B6	BP2	Dec 2006	5	No	30 (6)	0	0	1 (15)	Negative
B9	BP2	Jun 2007	12	No	30 (6)	0	0	nd	Negative
B6	BP2	Oct 2007	10	No	30 (6)	0	0	2 (15)	Negative
B5	BP3	Dec 2006	5	No	30 (6)	0	0	0 (15)	Negative
B5	BP3	Jun 2007	12	No	30 (6)	0	1	0 (15)	VHS
B3	BP3	Dec 2007	6	Yes	30 (6)	4	4	1 (15)	VHS
Total				2	360 (66)	4	5	4 (120)	
Programme 3									
B7	BP4	Jun 2007	13	Yes	15 (15)	2	2	nd	VHS
B7	BP4	Jul 2007	15	No	30 (6)	0	0	8 (30)	Negative
B6	BP4	Nov 2007	7	No	30 (6)	0	0	19 (30)	Negative
B6	BP4	May 2008	6	No	30 (6)	3	3	8 (30)	VHS
B10	BP4	Oct 2008	12	No	30 (6)	0	0	nd	Negative
B7	BP4E	Jul 2007	15	No	30 (6)	0	0	21 (30)	Negative
B6	BP4E	Nov 2007	7	No	30 (6)	0	0	16 (30)	IPN
B6	BP4E	May 2008	6	Yes	30 (6)	1	1	1 (30)	VHS
B10	BP4E	Oct 2008	12	No	30 (6)	0	0	nd	Negative
B8	BP5	Jun 2007	12	Yes	30 (6)	3	3	8 (30)	VHS, RTFS
B8	BP5	Jul 2007	15	No	30 (6)	0	0	25 (30)	Negative
B8	BP5	Nov 2007	6	No	242 (48)	0	0	14 (30)	Negative
B8	BP5E	Jul 2007	15	No	30 (6)	0	0	25 (30)	Negative
B8	BP5E	Dec 2007	6	Yes	200 (40)	1	1	2 (20)	VHS
Total				4	772 (169)	10	10	147 (350)	
Programme 4									
B12		Nov 2006	5	No	30 (3)	0	na	na	Negative
B4		Nov 2006	5	No	30 (3)	0	na	na	Negative
B8		Nov 2006	5	No	20 (2)	0	na	na	Negative
B14		Nov 2006	5	No	30 (3)	0	na	na	Negative

Table 2. (continued)

Sampled sites	Population ID	Sampling date	Temp. (°C)	Clinical signs	N fish sampled (N organ pools)	N positive organ pools Virus isolation	qRT-PCR	N seropositive fish ELISA+ (N tested fish)	Diagnosis
B15		May 2007	8	No	30 (3)	0	na	na	IPN
A1		Jun 2007	13	No	30 (3)	0	na	na	Negative
A1		Jun 2007	13	Yes	27 (2)	0	na	na	IPN, *Y.r.*
B9		Jun 2007	13	No	30 (3)	0	na	na	Negative
B5		Jun 2007	13	No	30 (3)	0	na	na	Negative
B1		Jun 2007	13	No	30 (3)	0	na	na	Negative
B12		Oct 2007	10	No	30 (3)	0	na	na	Negative
B13		Oct 2007	10	No	30 (3)	0	na	na	Negative
B14		Dec 2007	4	No	30 (3)	0	na	na	Negative
B14		Jun 2008	11	No	20 (3)	0	na	na	Negative
B5		Jun 2008	15	No	30 (3)	0	na	na	Negative
B12		Jun 2008	15	No	30 (3)	0	na	na	Negative
B13		Jun 2008	15	No	30 (3)	0	na	na	Negative
B15		Jun 2008	15	No	30 (3)	0	na	na	Negative
B11		Oct 2008	8	–	30 (3)	1	na	na	VHS
B9		Oct 2008	8	No	2 (1)	0	na	na	Negative
B12		Oct 2008	8	Yes	30 (3)	0	na	na	Negative
B12		Oct 2008	8	Yes	30 (3)	0	na	na	Negative
A1		Dec 2008	5	Yes	9 (3)	0	na	na	PKD
Total				5	618 (65)	1			

below this temperature were included in this study. VHS-diseased fish are often lethargic and dark in colour, with varying degrees of exophthalmia. Typical signs in the acute phase of the disease are widespread petechial haemorrhage, which can be seen throughout the internal organs, serosa, muscle tissue and eyes. In survivors, a chronic form of the disease may be seen when haemorrhaging is reduced, but anaemia may be severe. Swimming with an erratic and corkscrewing motion (termed 'flashing'), including surface swimming on some occasions, indicates a nervous stage of the disease (Smail & Snow 2011). There are no pathognomonic signs for VHS, and similar signs may also be seen in other infectious fish diseases. Therefore, the farmers were instructed to send in samples throughout the year. During the surveillance period (<15°C), the fish farmers sent in samples for autopsy 12 times from a total of 72 fish, which were combined into 42 organ pools (Table 2). No blood samples were collected from these fish.

Programme 2 was an active targeted surveillance programme focused on a population (AP2) from Company A and 2 populations (BP2 and BP3) from 2 farming sites (B6 and B5) of Company B. VHS disease had previously occurred at all of these farming sites, but only Company B was able to fallow its sites before surveillance started. The sample size was at least 30 fish population^{-1} on each sampling occasion, according to the official EU protocols 2001/183/EC and 2003/634/EC (EC 2001, 2003). Fish with clinical signs such as lethargy, dark skin, exophthalmia and erratic swimming indicating possible VHSV infection were first selected for sampling, and the rest were caught with a dip net. The fish were euthanized after capture, and individual blood samples were immediately collected from the caudal vein. The surveillance of AP2 started in June 2006, when the fish populations arrived from Denmark. The final samples were collected when the population was slaughtered during the winter of 2007. The surveillance of BP2 and BP3 started in December 2006, when the populations arrived from the VHS-free zone of continental Finland, and ended when the populations were slaughtered in December 2007. In Programme 2, the 3 selected populations (AP2, BP2 and BP3) were sampled 11 times altogether, and 360 fish were pooled into 66 organ samples (Table 2).

Programme 3 was also an active targeted surveillance programme. Two populations (BP4 and BP5) were selected from 2 fallowed farming sites (B7 and B8) of Company B immediately after a clinical VHS outbreak was diagnosed in these populations in June 2007. From these 2 farming sites, the fish populations next to the diseased populations BP4 and BP5 were also selected for surveillance. These 2 new populations, BP4E and BP5E, had no visible clinical signs of VHSV infection, but were potentially naturally exposed (E) to the virus. The surveillance started in June 2007 and ceased when the fish were slaughtered at the end of 2007 and 2008. Sampling was per-

formed as in Programme 2, with the exception that the first sample from BP4 included only 15 fish that were individually tested and the last samples from BP5 and BP5E included at least 200 fish. The VHSV-positive population BP4 and the neighbouring population, BP4E, were sampled 4 times after the first outbreak of clinical VHS in June 2007. Follow-up samples were taken in July 2007, November 2007, May 2008 and October 2008 from a total of 255 fish pooled into 63 organ pools (Table 2). The second VHSV-positive population, BP5, and its neighbouring population, BP5E, were sampled twice after the first outbreak of clinical VHS in June 2007, with 532 fish pooled into 106 organ pools (Table 2). Control fish of the same origin as BP4, BP4E, BP5 and BP5E were also tested. They were situated outside the VHS restriction zone.

Programme 4 comprised surveillance according to official EU protocols 2001/183/EC and 2003/634/EC (EC 2001, 2003), and was conducted at all farming sites of Companies A and B. The EU protocol requires that fallowed farming sites or sites where VHS has never been recorded are inspected twice a year and sampled once a year. VHS-positive farming sites were inspected once a year and sampled every second year during 2006–2008. Whole euthanized fish or organ samples (spleen, kidney and heart or brain) of 30 fish were sent to the Finnish Food Safety Authority Evira in Helsinki (the national reference laboratory) for virus isolation. Company A was sampled 3 times and samples were taken from a total of 66 fish pooled into 8 organ samples (Table 2). Company B was sampled 20 times from 9 different farming sites; altogether, 552 fish pooled into 57 organ samples were examined (Table 2). According to the legislation, fish farmers are obliged to inform authorities if they suspect notifiable diseases in their fish populations (2006/88/EC). Samples in connection with suspicion of disease were taken 4 times.

Virus isolation

Tissue samples of brain, anterior kidney and spleen were processed according to standard virological procedures described by Raja-Halli et al. (2006). The supernatant of the homogenized and centrifuged (4000 × *g*, 15 min) samples was collected for immediate inoculation into 24-well tissue culture plates (Nunc) with monolayer cell cultures of bluegill fry fibroblasts (BF-2) or epithelioma papulosum cyprinid (EPC) epithelial cells (Olesen & Vestergård Jørgensen 1992). In surveillance Programmes 1–3, the samples were inoculated in BF-2 and EPC cells no later than 24 h post-euthanasia. Virus isolation in Programme 4 was performed as in Programmes 1–3, with the exception that according to the instructions given in Commission Decision 2001/183/EC (EC 2001), the maximum time between euthanasia of the fish and inoculation of samples into cell culture was 48 h. All samples in all programmes were incubated in 2 passages for at least 14 d in total.

qRT-PCR for examining the presence of VHSV from tissue suspensions

qRT-PCR reactions were performed from samples in Programmes 1–3 according to Chico et al. (2006). A volume of 1 ml of the same organ suspension that was used for virus isolation was frozen at −80°C for qRT-PCR. RNA extraction was carried out using an RNeasy Mini Kit (Qiagen) starting with 200 µl suspension according to the manufacturer's protocol, and the final elution volume was 32 µl.

RT-PCR was performed with a QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Five microliters of extracted RNA was used in a 25 µl reaction volume. The final concentrations of the primers and the probe were 300 and 100 nM, respectively. The RT reaction profile was: 30 min at 50°C, 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

The primers and the probe for the qRT-PCR were manufactured (MedProbe) according to the VHSV sequence from GenBank accession no. D00687 after Chico et al. (2006). The probe was 5-end labelled with the fluorescent dye FAM and 3-end labelled with the fluorescent dye TAMRA (Table 3).

Sequencing of isolated VHSV strains

To confirm the presence of VHSV and to determine the genotype of the strain, the complete sequence of the glycoprotein (G) gene of 3 rainbow trout VHSV isolates from different surveillance programmes was amplified in 4 different RT-PCR reactions, and the amplicons were sequenced with primers used in the PCRs. RT-PCR amplifications were performed as described by Raja-Halli et al. (2006). Primer sequences (Table 3) targeting VHSV G, matrix (M) and non-structural (NV) gene regions were based on the published genome of VHSV strain Fi13 (Schütze et al. 1999).

Table 3. Primers and probe used for qRT-PCR in this study. For examining the presence of VHSV from tissue suspensions of samples collected in Programmes 1–3, qRT-PCR reactions were performed (following Chico et al. 2006) for which the primer sequence and position are based on viral haemorrhagic septicaemia virus (VHSV) mRNA for nucleocapsid (N) protein (GenBank accession no. D00687). To confirm the presence of VHSV and to determine the genotype of the strain, the complete sequence of the glycoprotein (G) gene of 3 rainbow trout VHSV isolates from different surveillance programmes was amplified in 4 different RT-PCR reactions, and the amplicons were sequenced with primers used in the PCRs. The primer sequences and positions are based on the genome of VHSV strain Fi13 (GenBank accession no. Y18263)

Primer name	Target gene	Forward primer	Position	Primer name	Target gene	Reverse primer	Position	Reference
47_QRT_F	N	5 CTCAACGGGACAGGAATGA	743–761	48_QRT_R	N	5 GGGCAATGCCCAAGTTGTT	791–809	Chico et al. (2006)
49_QRT_P	N	5 TGGGTTGTTCACCCAGGCCGC	769–789					Chico et al. (2006)
G1+	M	5 CGGGCAGGCGAAGGACTA	2768–785	G1–	G	5 TATCAGTCACCAGCGTCTCCG	3218–3238	Raja-Halli et al. (2006)
78	G	5 GGAATGGAACACTTTTTCTTGG	2961–2983	79	G	5 CACCAGGACCCATGATCACA	3636–3655	This study
88	G	5 CAACCTCGCCCTGTCAAACTC	3536–3556	89	G	5 TACGATGGGATGATATTTGGGGAC	4138–4161	This study
90	G	5 GACCCGGCAAGGCACACTAT	3983–4002	91	NV	5 TTTCTCCGCTCGTCCTCCG	4591–4609	This study

Sequencing reactions were performed using Big Dye v.3.1 chemistry (Applied Biosystems), and the reactions were run on an ABI3100 Avant genetic analyser. For each sample, the individual PCR sequences were edited and assembled into contigs by using Lasergene SeqMan Pro, v. 8.0.2 (DNASTAR). The GenBank accession numbers for the sequences reported herein are MF176925–MF176927.

The assembled sequences from this study were aligned with the G gene sequences of selected Finnish and other VHSV isolates belonging to genotypes I–IV (GenBank accession numbers AM086354, AM086358, AM086365, AM086379, AM086383, Z93 412, HQ112234, GQ504013, AY546582, AB179621), and the percentage identities between paired nucleotide sequences were calculated using the programme Megalign (DNASTAR).

Detection of antibodies against VHSV from trout serum with an indirect ELISA

Testing serum for antibodies against VHSV can reveal infected fish populations that could have been missed when testing organ samples for virus at an earlier point in time. Collected blood samples were centrifuged (3000 × *g*, 15 min) to obtain serum. The serum samples were heat inactivated for 30 min at 45°C (Olesen et al. 1991) and frozen (–80°C) until examination. The serum samples were tested for the presence of VHSV antibodies by an indirect ELISA method (diagnostic specificity, Sp: 1.0; diagnostic sensitivity, Se: 0.92) (Olesen et al. 1991). To verify the ELISA results, a set of samples was tested in parallel in another laboratory at Ploufragan/Plouzané, Unité de pathologie virale des poisons (Afssa). The samples were tested with the same ELISA method and with a serum neutralisation test (Sp 1.0 and Se 0.6) (Olesen & Vestergård Jørgensen 1986, Olesen et al. 1991, Castric et al. 2009). The received results were classified into positive/negative and were consistent with our results.

Statistical analysis

The effectiveness of different surveillance programmes in detecting VHSV infections was estimated using a binomial generalized linear model (GLM) (logit link):

$$\text{Logit}(Y) = a(\text{Programme1}) + b(\text{Programme2}) + c(\text{Programme3}) + d(\text{Programme4}) + eT + fT^2 \quad (1)$$

where Y = a positive detection of VHSV, T = temperature (°C) and a, b, c, d, e and f = coefficients. Programme 4 was treated as a reference category and was the intercept of the estimated model. An omnibus test was used to determine whether the model was better than the intercept-only model. A model without temperature as a covariate was estimated to assess whether the inclusion of temperature changed the relative efficiencies of the programmes. Probabilities of detection were calculated from a logistic model in the usual way: Probability of detection = $e^{(\text{relevant part of the GLM})}/[1+e^{(\text{relevant part of the GLM})}]$. For more information, see Dohoo et al. (2009). Statistical analyses were performed using IBM SPSS Statistics version 22.

The modified qRT-PCR test was compared with virus isolation by cell culture (gold standard test for detecting VHSV) in order to calculate the Se and Sp with Epitools (Sergeant 2016). The threshold cycle (C_t) cut-off was estimated using 2-graph receiver operating characteristic (TG-ROC) curves (Caraguel et al. 2011) with Epitools (Sergeant 2016).

RESULTS

Detection of VHSV infection in different programmes

Programme 1. VHSV was detected on 75% of the sampling occasions (Table 2). On 3 occasions, other infectious agents such as *Aeromonas salmonicida* sp. *salmonicida*, *Tetracapsuloides bryosalmonae* and *Flavobacterium psychrophilum* were diagnosed as the cause of the signs of disease. On 1 occasion, a mixed infection of VHS and *F. psychrophilum* was detected. On 9 sampling occasions, qRT-PCR was run from the same organ suspensions that were used for virus isolation, and the results were consistent with the virus isolation results (Table 2).

Programme 2. Population AP2, imported from Denmark in June 2006, was found to be infected by infectious pancreatic necrosis virus (IPN) at the time of import. IPN virus was neutralized from the samples with an anti-IPN serum to rule out a possible latent VHSV infection according to the OIE instructions (OIE 2017). VHSV was not detected in the imported fish at the time of arrival. Mortality in population AP2 caused by bacterial infection with *Yersinia ruckeri* was recorded in June 2007, but no evidence of the presence of VHSV was found either by virus isolation or qRT-PCR. Population BP2 was negative throughout the entire surveillance programme. In population BP3, VHSV was recorded twice. In June 2007, qRT-PCR gave for the first time a positive result for the presence of VHSV in the population, 6 mo after the beginning of the surveillance. VHSV was not isolated from these samples and no clinical signs of disease were noted by that time. In December 2007, at the time of slaughter, population BP3 experienced a clinical outbreak of VHS, and 4 out of 6 pools were positive for VHSV according to both virus isolation and qRT-PCR tests (Table 2).

Programme 3. VHSV was isolated again in BP4 in May 2008, about 1 yr after the first clinical infection when the surveillance started (Table 2). Population BP4E was also infected by that time and clinical signs of VHS were recorded. The second VHSV-positive population, BP5, and its neighbouring population, BP5E, were sampled twice after the first outbreak of clinical VHS in June 2007 (Table 2). No virus was detected and no clinical signs of infectious disease were noted in BP5 after June 2007. Mortality caused by VHSV was recorded in population BP5E in December 2007, 4 mo after the surveillance started. In BP5E, 1 out of 40 pools were positive according to both virus isolation and qRT-PCR. VHSV was not detected in the control fish.

Programme 4. VHSV was not detected in samples from Company A, although in 2 of 3 inspected populations, clinical symptoms of an infectious disease were reported to be present (Table 2). These symptoms were explained by *Y. ruckeri* infection, IPNV infection and proliferative kidney disease (PKD). VHSV was detected once in samples from Company B, but no information on whether these fish had clinical symptoms was available. Clinical symptoms were reported in 2 other screened populations, but in these cases, no VHS infection was detected and no additional sampling was performed to explain the symptoms. When the results for Companies A and B were combined, VHSV was detected on 4% of the sampling occasions (Table 2).

Efficiency of the sampling programmes in detecting VHSV by virus isolation

Both models (with and without temperature as a covariate) indicated that Programmes 1 and 3 gave positive results statistically significantly more often than Programme 4 (Table 4), whereas Programme 2 did not differ statistically significantly from Programme 4. This indicated that the probability of detection in these 2 surveillance programmes (2 and 4) was essentially very similar (Fig. 2). Depending on the

Table 4. Detection of VHSV by different surveillance programmes, estimated using a binomial generalized linear model (GLM), where temperature was included or excluded as a covariate. The estimated coefficient and the standard error (SE) are given, as well as Wald test statistics and the corresponding probability estimate that H_0 (coefficient is equal to 0) would be true

	Coef. (SE)	Wald	p
Model with temperature			
Programme 1	5.19 (1.14)	20.83	0.000
Programme 2	0.76 (1.44)	0.27	0.600
Programme 3	2.87 (1.22)	5.52	0.019
Programme 4 (intercept)	−7.73 (2.58)	8.97	0.003
T	1.41 (0.64)	4.89	0.027
T^2	−0.09 (0.04)	6.07	0.014
Model without temperature			
Programme 1	4.19 (1.22)	11.78	0.001
Programme 2	0.79 (1.47)	0.29	0.590
Programme 3	2.40 (1.16)	4.27	0.039
Programme 4 (intercept)	−3.09 (1.02)	9.14	0.003

model, Programme 1 gave a 13.7–17.4 times higher probability of detection and Programme 3 a 7.7–8.3 times higher probability of detection than Programme 4 (the official EU standard) (Fig. 2).

As can be seen from Table 4, the standard errors of the coefficients were slightly higher in the model that did not take into account the temperature effect on VHSV detection. However, since the parameter uncertainty of the covariates (T and T^2) also increased the confidence intervals of the probability of detection, the benefit of the more complicated model appeared to be small in this case (Table 4). Nonetheless, temperature seemed to have a statistically significant impact on model performance.

Detection of VHSV antibodies in screened populations

Antibodies against VHSV were detected in only 4 serum samples out of 120 tested in Programme 2 (Table 2). In Programme 3, populations BP4, BP4E, BP5 and BP5E tested positive for VHSV antibodies on several occasions (Table 2).

Correspondence of diagnostic methods on each sampling occasion

The C_t cut-off for the qRT-PCR method that was used was set at 36. qRT-PCR analysis for separate sampling occasions (Programmes 1–3) corresponded well with

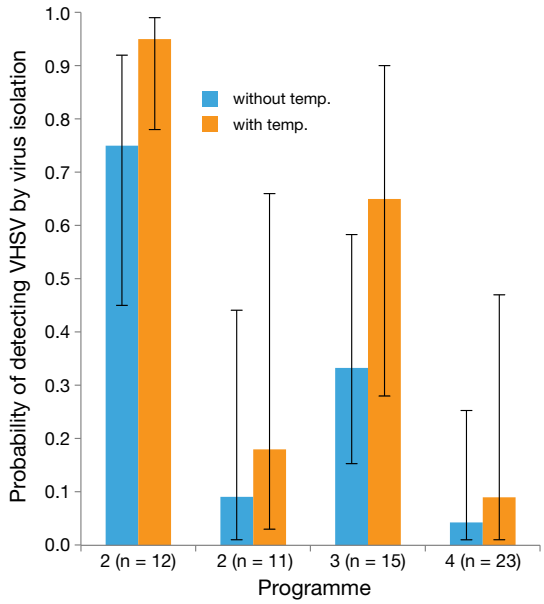

Fig. 2. Probability of detecting viral haemorrhagic septicaemia virus (VHSV) by virus isolation in different surveillance programmes (see Table 1) estimated using a generalized linear model (GLM) that included ('with temp.') or excluded ('without temp.') temperature as a covariate. Marginal means were adjusted to 10.0°C if temperature was applied in the GLM. Error bars represent the 95% confidence interval for the mean. The number of samples in each programme is given in parentheses

the virus isolation results, as the kappa value was 0.877. The sensitivity and specificity of qRT-PCR was 1 and 0.959, respectively. The positive likelihood ratio was 24 and the negative likelihood ratio 0. Confidence limits were 0.92–1.00 for sensitivity and 0.92–0.98 for specificity.

Sequencing of isolated VHS viruses

The G gene of 3 rainbow trout VHSV isolates (Fi08.50RT, Fi06.59RT and Fi06.108RT) originating from different surveillance programmes in this study was sequenced and compared with the G gene sequences of some Finnish and other VHSV isolates belonging to genotypes I–IV (Table 5, Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d126p111_supp.pdf). Pairwise sequence comparisons showed that the rainbow trout isolates of this study were most closely related to the Finnish rainbow trout genotype I isolates from Åland, isolated during

Table 5. Reference viral haemorrhagic septicaemia virus (VHSV) isolates used in the sequence comparisons

VHSV isolate	Host	Location and year of isolation	Genotype	GenBank acc. no.
Fi01a_b.00	Rainbow trout	Finland 2000	Id	AM086354
FiA02a.01	Rainbow trout	Finland 2001	Id	AM086358
FiA03.02	Rainbow trout	Finland 2002	Id	AM086365
FiA03.03	Rainbow trout	Finland 2003	Id	AM86379
Fi19.04	Rainbow trout	Finland 2004	Id	AM086383
Hededam	Rainbow trout	Denmark 1972	I	Z93412
FI-ka366-04	Herring	Finland 2004	II	HQ112234
FI-Lamprey-743.03	Lamprey	Finland 2003	II	GQ504013
DK-4p168	Herring	Skagerrak 1997	III	AY546582
KRRV9822	Japanese flounder	Japan 2000	IV	AB179621

2000–2004 (Raja-Halli et al. 2006). The nucleotide (nt) identity between these isolates was 99.7–99.9%, whereas the nt identity between Finnish lamprey and herring VHSV isolates belonging to genotype II (Gadd et al. 2010, 2011) and isolates from this study was only approximately 89% (Table S1 in the Supplement). The G gene sequence identity between VHSV isolates from this study and VHSV isolates belonging to genotypes III and IV was approximately 91 and 86%, respectively. These results indicate that the current VHSV isolates belonged to genotype I and confirm that VHS infection was still present in Åland during the time of this study.

DISCUSSION

Syndromic surveillance (Programme 1) was the most reliable means to screen for the presence of VHSV, being up to 17 times more effective than active surveillance in Programme 4 (Table 4). This result indicates that active surveillance only using methods to detect virus, although regarded as sufficient to show infection in a zone, is not a reliable tool to reveal whether a single population is or has been infected by VHSV. It is notable that Programme 3, with sampling after a clinical VHS outbreak, performed almost as well as Programme 1, while the other 2 sampling programmes (2 and 4) performed less efficiently. In practice, an active surveillance scheme that is performed after a confirmed clinical VHS outbreak would not be a sensible strategy to identify VHSV-infected farms, as they will have already been found. In contrast, syndromic surveillance outperformed active surveillance programmes and has clear practical value.

Both virus isolation in cell culture and qRT-PCR are reliable tests to detect VHSV when there is an acute VHSV infection at the time of sampling, but effective early warning systems are required to detect signs of the disease. qRT-PCR is a rapid and reliable test to confirm or rule out the presence of VHSV in organ suspension when clinical signs have given reason to suspect infection. qRT-PCR is also reported to be valuable in finding asymptomatic fish carrying VHSV (Hope et al. 2010). In our study, qRT-PCR detected a possible carrier on 1 occasion when virus isolation in cell culture failed. This was a situation in spring 2007 when the water temperature was close to 15°C and rising. It is possible that this population had just been infected and clinical signs of VHS had not appeared before the water temperature rose above 15°C. We have noticed that outbreaks due to VHSV Id do not occur and the virus cannot be isolated at temperatures higher than 15°C. Serum samples that were collected on the same occasion did not reveal any antibodies against VHSV, also suggesting an early infection. Our suspicion concerning the carrier state was confirmed the next autumn when the water temperature dropped below 15°C and the fish in this population experienced a clinical disease outbreak. Serum samples taken at this time revealed only 1 positive sample out of 15 tested. This suggests a new infection and indicates that the virus infection did not have time to spread in the population before water temperature rose above 15°C during the previous spring.

qRT-PCR is valuable when screening for a particular virus, e.g. in wild fish, and where a positive signal does not lead to legal actions against the business owner. qRT-PCR could also be used as the primary diagnostic screening test for fish farms, but a positive result should be confirmed by other methods such as antibody testing. ELISA or plaque neutralisation tests have been reported to be good tools to screen for antibodies against VHSV in Europe and the USA (Fregeneda-Grandes & Olesen 2007, Fregeneda-Grandes et al. 2009, Schyth et al. 2012, Millard et al. 2014, Wilson et al. 2014). Our study also proved ELISA to be a useful method for screening, since VHSV infection could be detected several months after a clinical disease outbreak occurred. However, the results were only easy to interpret if there had been a clear clinical outbreak no more than 1 yr previously in the population.

The water temperature in the sea area in Finland varies from slightly above 0°C in winter to often above 20°C in summer. In autumn, the water temper-

ature drops below 15°C in late September, and ice usually covers the farming sites from December to late April. This ice layer makes inspection and sampling impossible for several months. When the ice layer melts in spring, the water temperature often rises above 15°C within 2–3 mo, varying from year to year. This gives a short time window for the authorities to visit the farms, which are scattered around thousands of small, difficult to reach islands. Additionally, the manpower of the authorities is restricted, as they have duties covering all animal groups and usually limited experiences of fish diseases.

Early detection of VHS is essential for successful disease eradication. Virus infections are easily spread between farms in the same area due to daily management. Routine clinical inspections performed by skilled fish health specialists have also been noted as essential in the surveillance of freedom from VHS in Norwegian marine salmon farms (Lyngstad et al. 2016). Our study supports the Norwegian report by demonstrating that more frequent monitoring for clinical signs of VHS outperforms active surveillance. There are no fish health services offering routine clinical inspection or sampling services for the fish farms in the study area. The farming sites are difficult to reach and shipping of samples by the farmers themselves for testing of diseases is complicated, as the logistics involved in transferring samples between the study area and the laboratory are poor. Farmers only sent samples when high mortalities were seen; mild signs of disease were often not confirmed. VHSV Id was reported to cause 40% mortalities in an infection trial (Raja-Halli et al. 2006), but we have rarely seen such high mortalities in the study area (authors' unpubl. data). Mortalities ranging from 10 up to 50% have only been seen under stressful conditions, such as the transportation of infected populations between farming sites.

The good results of syndromic surveillance in Programme 1 could have been because the staff of the fish farm already had 6 yr of experience with VHS and good skills in detecting abnormalities in a fish population indicating a disease outbreak in the early stages of infection. The finding that temperature affected the performance of the programmes might be associated with this; fish farmers found the occurrence of clinical cases at certain temperatures to be typical of the disease. The staff of the study fish farms were also motivated to participate in this surveillance, and new means of transportation of samples to the laboratory were found, which was considered to be vitally important for this field study. The populations were also carefully observed at the time of sampling for screening in Programmes 2 and 3, but only the farmers were able to follow up their fish populations daily. Another explanation for the good results in Programme 1 could be that this type of screening finds infections in their early stages, when it is easier to detect the virus because of the higher viral amounts in the samples. Sandlund et al. (2014) reported that gills are useful target organs in screening chronic or sub-acute VHSV infections. Therefore, it could be argued that Programme 1 would not differ as much from the other programmes if gills had also been tested in the other programmes. However, gills were used on 2 occasions in parallel with the other organ samples of the same fish in this study without gaining any new information (P. Vennerström unpubl. data). Additionally, it could be argued that Programme 4 would have been more efficient if farmers had reported their suspicions according to the legislation. It is doubtful that VHSV would have been diagnosed to the same extent without the separate Programme 1. The successful eradication of VHSV in 2 other areas on the south coast of Finland in 2001 and 2003 (reinfection 2008) could be explained by the early detection and rapid eradication of affected farming sites, which is vital for the eradication of and subsequent freedom from disease. Farmers contacted the authorities immediately when they noticed suspicious disease signs. Eradication was performed without delay and in good cooperation between farmers and authorities. In Åland, this cooperation was not as successful at the beginning and VHSV rapidly spread between farming sites, and stamping out the disease was not economically justified. We believe that this study managed to improve the screening of VHSV and biosecurity measures in this area. According to the official disease surveillance in the restriction area in Åland, the number of VHS-positive samplings has followed a decreasing trend (ICES 2014), which indicates a lower infection pressure in the area. VHSV has not been isolated in the study area since 2012 (Finnish Food Safety Authority 2016).

CONCLUSION

Syndromic surveillance (based on the observation of clinical disease signs by fish farmers) is more sensitive than active surveillance when detecting VHSV infections on fish farms. Active surveillance (Programmes 2, 3 and 4) did not yield information that would have been needed for preparing eradication plans in the VHS eradication zone of Åland.

Real-time RT-PCR was at least as reliable as virus isolation in cell culture to detect infection in this study, but serology proved to be a useful test to determine whether a fish population had been infected with VHSV. However, the antibody levels are very low in mild disease outbreaks or if the infection has occurred several months earlier. Therefore, the use of this antibody test in screening for VHSV in disease-free areas is not reliable without affirmation of the test results with another test, such as virus isolation or PCR. On the other hand, it is a useful additional tool in VHSV eradication for screening populations during the follow-up period, before declaring an area free of infection.

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Wild fish are negligible transmitters of viral haemorrhagic septicaemia virus (VHSV) genotype Id in the VHS restriction zone in Finland

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ABSTRACT: Wild fish were suspected to be the source of reinfection by viral haemorrhagic septicaemia virus (VHSV) in Finnish brackish water rainbow trout farms located in a restriction zone regarding viral haemorrhagic septicaemia (VHS) comprising the entire Province of Åland, Baltic Sea, in the 2000s. Altogether, 1636 wild fish of 17 different species living in the vicinity of infected fish farms were screened for VHSV during the years 2005–2008. Additionally, 2 uninfected wild fish species as well as farmed whitefish were introduced into a VHS-positive fish farm to test whether they became infected by VHSV from the clinically diseased rainbow trout. Wild fish did not test positive for VHSV on any occasion. In contrast, whitefish introduced to a VHS-positive farm were infected with VHSV genotype Id and started to replicate the virus for a short time during the trial. Whitefish are farmed together with, or in the vicinity of, farmed rainbow trout in the study area and, according to this study, are a possible source of the recurring infection in the restriction area. A sprivivirus was isolated from all fish species in the infection trial without causing mortality in the test groups.

KEY WORDS: Viral hemorrhagic septicemia · VHSV · Aquaculture · Disease control · Whitefish · Wild fish · Carrier · Epidemiology

INTRODUCTION

Viral haemorrhagic septicaemia viruses (VHSVs) are a group of novirhabdoviruses that has been isolated from a wide range of wild and farmed fish species in Europe, North America and Japan living in both marine and freshwater (review by Skall et al. 2005, Elsayed et al. 2006, Lumsden et al. 2007, Dale et al. 2009, Bain et al. 2010, Gadd et al. 2010, 2011, Kim & Faisal 2010, Emmenegger et al. 2013, Ito & Olesen 2013, OIE 2017). VHSV is a negative-stranded RNA virus of which isolates cluster into 4 different genotypes (I–IV), with several subtypes in genotypes I (Ia–Ie) and IV (IVa–IVd) (Snow et al. 1999, Einer-Jensen et al. 2005, Elsayed et al. 2006, Ammayappan & Vakharia 2009, Pierce & Stepien 2012, Cuenca et al. 2017). The genotypes are generally more tied to geographical region than to target species (Snow et al. 1999, Nishizawa et al. 2002, Thiery et al. 2002, Einer-Jensen et al. 2004).

According to phylogenetic studies, VHSV has its ancestors in the marine environment, from where it has adapted to be a serious disease agent for farmed rainbow trout *Oncorhynchus mykiss* (Einer-Jensen et

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al. 2004). The 'change in host range' has probably happened several times since the first reports of clinical outbreaks of viral haemorrhagic septicaemia (VHS) originating from the 1950s (Einer-Jensen et al. 2004). These historical leaps over the species barriers are likely, at least in large part, attributed to human activities in connection with fish farming procedures and virus adaptation to new hosts. Wild marine fish, mainly herring *Clupea harengus*, were intensively used as minced fresh feed for freshwater farmed rainbow trout in Europe in the 1950s (Meyers & Winton 1995, Dixon 1999, Einer-Jensen et al. 2004). The use of fresh marine fish as a source of feed decreased throughout Europe when several wild marine fish species, including herring, were identified as carriers of VHSV and it was found that VHSV could be spread by the oral route (Stone et al. 1997, Snow et al. 1999, Mortensen et al. 1999). VHSV types pathogenic to rainbow trout have been isolated from marine wild fish in Europe on some occasions, but no mass mortality or clinical signs of VHS have been reported in these wild fish species (Skall et al. 2005). In contrast, in North America, mortalities due to infections with VHSV have been reported on several occasions in wild fish (Marty et al. 1998, Meyers et al. 1999, Elsayed et al. 2006, Gagné et al. 2007, Groocock et al. 2007, Lumsden et al. 2007, Ammayappan & Vakharia 2009).

In Finland, VHS has been reported in 3 separate fish farming locations on the south and southwest coasts during 2000–2012 (Raja-Halli et al. 2006). VHS was first isolated in 2000 on the southwest coast of Finland in the Province of Åland and a few weeks later on the south coast. VHSV was isolated at a third location also on the west coast in 2003 and again in 2008 (Raja-Halli et al. 2006, Finnish Food Safety Authority 2010). Mortalities in these disease outbreaks have varied from negligible up to 50% on some occasions. VHS in fish farms in 2 of these areas has been successfully eradicated, but the area where VHS was first recorded in the Province of Åland is still classified as VHS-positive. A common source of the outbreaks in Finnish fish farms is suspected as all of the VHSV isolates were genotype Id and, according to sequencing results, almost identical (Raja-Halli et al. 2006). The source of the first VHS outbreak in Finland is still unknown, but wild fish, mainly herring, are considered to be the most likely source of infection since VHSV genotype Id (hereafter VHSV Id) is genetically close to isolates from wild fish and early Danish isolates from farmed rainbow trout (Skall et al. 2004). Further evidence supporting herring as a likely source is the fact that wild herring had previously been used as minced fresh feed in some fish farms in this area (Raja-Halli et al. 2006).

Wild salmonid brood fish, Baltic herring *Clupea harengus membras* and lamprey *Lampetra fluviatilis* have been screened for VHSV in the same parts of the Baltic Sea where these VHSV-positive Finnish fish farms are located, but VHSV Id has not been isolated from wild fish. Nonetheless, Baltic herring and lamprey were found to be carriers of VHSV genotype II (Gadd et al. 2010, 2011). The highest prevalence of VHSV II, 50 of 479 pools (10.4%), was recorded in herring originating from the Archipelago Sea southwest of Finland (Gadd et al. 2011). However, VHS has never been recorded in farmed fish in this area even though intensive fish farming has been performed for decades and minced wild herring was previously used as fresh feed. According to the infection trials, the pathogenicity of the isolated VHSV II strains to rainbow trout was negligible (Gadd et al. 2011).

The aim of the present study was to survey whether wild fish living in close proximity to the net pens in VHSV-positive fish farms could be carriers of this virus. Additionally, the aim was to study under field conditions whether VHSV Id can be naturally transmitted to perch *Perca fluviatilis*, roach *Rutilus rutilus* and whitefish *Coregonus lavaretus* from clinically diseased farmed rainbow trout in a VHSV-positive fish farm.

MATERIALS AND METHODS

Collection of wild fish for virus examination

Wild fish were caught in the immediate vicinity of 2 VHSV-positive food fish farms that were farming rainbow trout in the brackish sea area in the Baltic Sea; fish were caught beside, between and under the net pens. The farms are situated in the Province of Åland, in a VHS restriction zone that was established in 2001 after VHSV was detected in several farms (Raja-Halli et al. 2006). The fish were caught with 2 specially designed nets with 4 different mesh sizes (10, 20, 30 and 40 mm) on 12 different occasions: once in autumn 2005, 6 times in spring 2006, 3 times in autumn 2006, once in spring 2007 and once in autumn 2008. The nets were set in the water in the vicinity of the net pens in the evening and kept in place overnight before they were pulled up from the water and emptied of fish. Species caught and the number of different species tested are presented in

Table 1. Rainbow trout in both farms in the restriction zone tested VHSV-positive on several occasions before the sampling, and on 2 occasions in spring 2006 the sampling was performed during a clinical VHS outbreak on the nearby fish farm. All caught wild fish were tested, unless over 100 fish per species were caught, in which case only 100 fish of those species were collected non-randomly for testing. Tissue samples from the anterior kidney, spleen, brain and heart were taken and samples from a maximum of 5 fish were pooled together. Fish species that rapidly decompose due to high fat content, such as herring and sprat, were sampled immediately after capture, and the rest of the fish were iced and transported to the laboratory for further sampling. All tissue samples were obtained and appropriately preserved within 1 d of capture. The water temperature ranged from 2°C to 13°C during the sampling.

Table 1. Laboratory results for surveillance of viral haemorrhagic septicaemia virus (VHSV) for 17 wild fish species in the vicinity of 2 VHSV-positive fish farms in the viral haemorrhagic septicaemia restriction zone of the Province of Åland, in the Baltic Sea, during 2005–2008. qRT-PCR: real-time reverse transcription PCR; PS: positive strand; NS: negative strand; nd: not determined; CI: confidence interval

Fish species	Number of fish sampled				Results for laboratory examinations				Prevalence (%)	
	Autumn	Spring	Total	Percentage	Virus isolation	qRT-PCR	PS qRT-PCR	NS qRT-PCR	Observed	Median estimate (95% CI)
Perch *Perca fluviatilis*	300	213	513	31.36	Negative	Negative	nd	nd	0	0.04 (0–0.49)
Bleak *Alburnus alburnus*	70	329	399	24.39	Negative	Negative	nd	nd	0	0.06 (0–0.63)
Roach *Rutilus rutilus*	168	97	265	16.20	Negative	Negative	nd	nd	0	0.09 (0–0.94)
Three-spined stickleback *Gasterosteus aculeatus*	2	151	153	9.35	Negative	Negative	nd	nd	0	0.15 (0–1.63)
Ruffe *Gymnocephalus cernuus*	0	87	87	5.32	Negative	Negative[a]	Negative	Negative[a]	0	0.26 (0–2.84)
Herring *Clupea harengus membras*	30	46	76	4.65	Negative	Negative[b]	Negative	Negative	0	0.30 (0–3.27)
White bream *Abramis bjoerkna*	30	36	66	4.03	Negative	Negative	nd	nd	0	0.75 (0–3.72)
Smelt *Osmerus eperlanus*	0	32	32	1.96	Negative	Negative	nd	nd	0	1.51 (0–7.49)
Rudd *Scardinius erythrophthalmus*	4	16	20	1.22	Negative	Negative	nd	nd	0	1.12 (0–11.66)
Rainbow trout *Oncorhynchus mykiss*	1	6	7	0.43	Negative	Negative[c]	Negative	Negative	0	3.09 (0–29.24)
Fourhorn sculpin *Triglopsis quadricornis*	2	4	6	0.37	Negative	Negative[d]	nd	nd	0	3.57 (0–33.04)
Flounder *Platichthys flesus*	1	4	5	0.31	Negative	Negative	nd	nd	0	4.23 (0–37.94)
Sea trout *Salmo trutta*	0	2	2	0.12	Negative	Negative	nd	nd	0	9.55 (0–66.68)
Straight-nosed pipefish *Nerophis ophidion*	2	0	2	0.12	Negative	Negative	nd	nd	0	9.55 (0–66.68)
Eelpout *Zoarces viviparus*	0	1	1	0.06	Negative	Negative	nd	nd	0	16.3 (0–85.33)
Ide *Leuciscus idus*	1	0	1	0.06	Negative	Negative	nd	nd	0	16.3 (0–85.33)
Pike *Esox lucius*	0	1	1	0.06	Negative	Negative	nd	nd	0	16.3 (0–85.33)
Total	611	1025	1636	100.00						

[a–d]Organ suspensions (pools) that gave a weak signal with qRT-PCR threshold cycle (C_t) >36: [a]1 out of 17 pools, [b]1 out of 20 pools, [c]1 out of 7 pools and [d]1 out of 4 pools

Infection trial in field conditions

Altogether, 500 wild perch *Perca fluviatilis* and roach *Rutilus rutilus* were caught with a bow net on the southwest coast of Finland, outside the VHS restriction zone. To determine whether the wild-caught fish were free of VHSV, control samples of 100 fish of each species were non-randomly collected with a dip net for virus testing. Of each species, 150 live fish were transported in 1500 l aerated transport tanks to a VHSV-positive fish farm in the VHS restriction zone in the Province of Åland. Additionally, 150 whitefish from a fish farm in the VHS-free continental zone in Finland were transported in the same way to the same farm. Control samples of 40 whitefish were collected non-randomly with a dip net for virus testing before transport. The fish were placed in 9 small net cages, with 50 fish in each cage and 3 cages per species. The test cages were placed beside the sea cages where clinical VHS had been confirmed in rainbow trout 1 wk earlier. Fifty fish of each species functioned as control groups in similar cages but at another fish farm in the sea area outside the VHS restriction zone. The fish were inspected every day and 10 fish per cage were collected for sampling on Days 10, 14, 21 and 35 after the transfer (Table 2). Fish with any signs of disease or that were in poor condition were first selected for sampling; otherwise, fish were picked non-randomly with a dip net for sampling. The rainbow trout at the farms were also tested for VHSV on Days 0, 10 and 14. After Day 14, the rainbow trout in the farm were moved to a nearby summer farming site and only the test cages were left on the farm.

All fish that were collected for testing for the presence of VHSV were transported on ice to the laboratory and sampled the next day. Tissue samples from the brain, spleen, heart and anterior kidney of 5 fish from the same cage were pooled together (2 pools per cage). Samples from the gills and skin were also collected from these fish and pooled in 2 separate tissue pools per cage. The control cages at the control

Table 2. Results from the infection trial performed to reveal whether wild perch, roach and farmed whitefish could be infected by viral haemorrhagic septicaemia virus (VHSV) in field conditions at a VHSV-positive fish farm in the viral haemorrhagic septicaemia restriction zone of the Province of Åland in the Baltic Sea. qRT-PCR: real-time reverse transcription PCR; PS: positive strand; NS: negative strand; nd: not determined. VHSV-positive pools are highlighted in **bold**

Day of sampling	Temp. (°C)	Method	Number of VHSV-positive organ, gill and skin pools/number of pools tested												
			Perch			Roach			Whitefish			Farmed rainbow trout	Controls (perch, roach and whitefish) – combined results[a]		
			Organs	Gills	Skin	Organs	Gills	Skin	Organs	Gills	Skin	Organs	Organs	Gills	Skin
0	4	Virus isolation	0/20	nd	nd	0/20	nd	nd	0/8	0/3	0/3	**8[b]/8**	0/9	nd	nd
		qRT-PCR	0/20	nd	nd	0/20	nd	nd	0/8	0/3	0/3	**8/8**	0/9	nd	nd
		PS qRT-PCR	nd	nd	nd	nd	nd	nd	nd	nd	nd	**8/8**	nd	nd	nd
		NS qRT-PCR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	6	Virus isolation	0/6	0/3	0/3	0/6	0[c]/3	0/3	0/6	0/3	0/3	**4/4**	0/12	0/12	0/12
		qRT-PCR	0/6	0/3	0/3	0/6	1[d]/3	0/3	**1/6**	**1/3**	**1/3**	**4/4**	0/12	0/12	0/12
		PS qRT-PCR	nd	nd	nd	nd	0/6	nd	0/6	0/6	0/6	**4/4**	nd	nd	nd
		NS qRT-PCR	nd	nd	nd	nd	0/6	nd	nd	nd	nd	**4/4**	nd	nd	nd
14	7	Virus isolation	0/6	0/3	0/3	0/6	0/3	0/3	**1[b]/6**	**1[b]/3**	**1[b]/3**	**4/4**	0/12	0/12	0/12
		qRT-PCR	0/6	0/3	0/3	0/6	0/3	0/3	**1/6**	**1/3**	**1/3**	**4/4**	0/12	0/12	0/12
		PS qRT-PCR	nd	nd	nd	nd	nd	nd	**1/6**	**1/3**	**1/3**	**4/4**	nd	nd	nd
		NS qRT-PCR	nd	nd	nd	nd	nd	nd	**1/6**	**1/3**	**1/3**	**4/4**	nd	nd	nd
21	9	Virus isolation	0/6	0/3	0/3	0[c]/6	0[c]/3	0[c]/3	0/6	0/3	0/3	nd	0/12	0/12	0/12
		qRT-PCR	0/6	0[c]/3	0/3	0/6	0/3	0/3	0/6	0/3	0/3	nd	0/12	0/12	0/12
		PS qRT-PCR	nd	nd	nd	nd	nd	nd	0/6	0/3	0/3	nd	nd	nd	nd
		NS qRT-PCR	nd	nd	nd	nd	nd	nd	0/6	0/3	0/3	nd	nd	nd	nd
35	11	Virus isolation	0/6	0/3	0/3	0[c]/6	0[c]/3	0[c]/3	0/6	0[c]/3	0/3	nd	0/12	0/12	0/12
		qRT-PCR	0/6	0/3	0/3	0/6	0/3	0/3	0/6	0/3	0/3	nd	0/12	0/12	0/12
		PS qRT-PCR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		NS qRT-PCR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a]Three pools per fish species: 1 pool of organs from 5 fish, and 1 pool of gills and 1 pool of skin collected from 10 fish
[b]Isolates that were sequenced: Fi08.50RT, Fi08.22WF, Fi08.23RT, Fi08.24RT (see Table 4)
[c]A sprivivirus was isolated from all fish species, mostly in roach
[d]Samples gave a weak VHSV signal with qRT-PCR threshold cycle (C_t) >36. VHSV-positive (C_t < 36) pools are highlighted in bold

farm were inspected and sampled likewise. The sampling regime is presented in Table 2.

Virus isolation

Tissue samples of the brain, spleen, heart and anterior kidney from wild fish and fish from the infection trial were examined for the presence of VHSV according to EU Commission Decision 2001/183/EEC (EC 2001) (see Tables 1 & 2). Organ samples from a maximum of 5 fish were pooled in 9 volumes of cell culture medium (Eagle's minimum essential medium, Gibco, and 10% fetal bovine serum, pH 7.4) containing penicillin and streptomycin. Gill and skin samples were examined separately in pools consisting of 10 fish. All samples were homogenized and centrifuged (15 min at 4000 × *g*, 4°C), and each organ pool was treated separately to avoid contamination between samples. The supernatant was collected for immediate inoculation into 24-well tissue culture plates (Nunc) with monolayer cell cultures of bluegill fry (BF-2) fibroblasts and epithelioma papulosum cyprinid (EPC) epithelial cells (Olesen & Jørgensen 1992). The samples were inoculated in BF-2 and EPC cells no later than 24 h post euthanasia. All samples were incubated in 2 passages for a total of at least 14 d. Isolated virus strains were confirmed to be VHSV with a commercial ELISA kit according to the manufacturer's instructions (TestLine) and some isolates of VHSV were confirmed with additional sequencing.

Real-time RT-PCR for examining the presence of VHSV from tissue suspensions

One microlitre of the same tissue suspension that was used for virus isolation was frozen in −80°C to be later tested with a real-time reverse transcription polymerase chain reaction (qRT-PCR) (later called diagnostic qRT-PCR). RNA extraction was carried out using an RNeasy Mini Kit (Qiagen), and qRT-PCR was performed with a QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The final concentrations of the primers and probe were 300 and 100 nM, respectively. The reverse transcription reaction profile was 30 min at 50°C and 15 min at 95°C; followed by 50 cycles of 15 s at 95°C and 1 min at 60°C.

The primers and the probe (Med Probe) for the diagnostic qRT-PCR were manufactured according to the VHSV N gene sequence from GenBank accession number D00687 after Chico et al. (2006). The probe was 5′-end labelled with FAM and 3′-end labelled with TAMRA (Table 3). We used a threshold cycle (C_t) cut-off value of 36 as estimated in our earlier study (Vennerström et al. 2017).

Strand-specific qRT-PCR

As sampling was performed in an environment assumed to be highly contaminated by VHSV, a new qRT-PCR method (hereafter, strand-specific qRT-PCR) was developed to test for negative and positive stranded VHSV products formed during VHSV replication. This method could be used to rule out possible environmental virus contamination and to test whether the virus was actively replicating in the tested fish tissues. This strand-specific qRT-PCR method is based on a method previously described by Purcell et al. (2006). Controls for the strand-specific method were created with *in vitro* transcription from cloned VHSV N gene amplicons. Primers and probes used are presented in Table 3.

Cloning of PCR product for *in vitro* transcription

To create a template for positive and negative control RNA, an 810 bp amplicon from VHSV N gene PCR was inserted into a pSC-A plasmid and transfected into StrataClone SoloPack competent cells (StrataClone™ PCR Cloning Kit, Stratagene) according to the manufacturer's instructions. The resulting plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen) and verified by restriction digestion and sequencing with the universal T3 and T7 primers. Sequencing was performed by the Institute of Biotechnology, University of Helsinki, Finland.

In vitro transcription

The control RNA for strand-specific qRT-PCR was prepared in 2 separate *in vitro* transcription reactions to produce both positive and negative strand RNA. Based on sequencing, the orientation of the insert could be determined, and linearization of the plasmid was performed with *Bam*HI (Fermentas) or *Hin*dIII (Fermentas) for positive- or negative-strand RNA transcription, respectively.

In vitro transcription of linearized plasmids was performed with a MAXIscript T7/T3 Transcription Kit (Ambion, Applied Biosystems) according to the manufacturer's instructions. Both positive- and negative-

Table 3. Primers and probes used for real-time reverse transcription PCR (qRT-PCR), strand-specific qRT-PCR and sequencing for examining the presence of viral haemorrhagic septicaemia virus (VHSV) from tissue samples collected from wild fish, farmed fish and fish from an infection trial in the viral haemorrhagic septicaemia restriction zone of the Province of Åland, in the Baltic Sea. Primer sequence and position for diagnostic and strand-specific qRT-PCR are based on VHSV mRNA for nucleocapsid (N) protein (GenBank accession number D00687). The primer sequences and positions for sequencing are based on the genome of VHSV strain Fi13 genome (GenBank accession number Y18263)

Primer name	Target gene	Forward primer	Position	Primer name	Target gene	Reverse primer	Position	Reference
Diagnostic qRT-PCR								
47_QRT_F	N	5′-CTCAACGGGACAGGAATGA	743–761	48_QRT_R	N	5′-GGGCAATGCCCAAGTTGTT	791–809	Chico et al. (2006)
49_QRT_P	N	5′-TGGGTTGTTCACCCAGGCCGC	769–789					Chico et al. (2006)
RT-PCR before cloning								
41	N	5′-GGGGACCCCAGACTGT	261–276	42	N	5′-TCTCTGTCACCTTGATCC	1053–1070	Present study
Strand-specific qRT-PCR								
67_Tag_F/R	Tag	5′-GGCAGTATCGTGAATTCGATGC						Purcell et al. (2006)
68_Tag_47_F	N	5′-GGCAGTATCGTGAATTCGATGC CTCAACGGGACAGGAATGA	743–761	69_Tag_48_R	N	5′-GGCAGTATCGTGAATTCGATGC GGGCAATGCCCAAGTTGTT	791–809	Purcell et al. (2006), Chico et al. (2006)
RT-PCR / sequencing								
G1+	M	5′-CGGGCAGGCGAAGGACTA	2768–785	G1–	G	5′-TATCAGTCACCAGCGTCTCCG	3218–3238	Raja-Halli et al. (2006)
78	G	5′-GGAATGGAACACTTTTTTCTTGG	2961–2983	79	G	5′-CACCAGGACCCATGATCACA	3636–3655	Present study
88	G	5′-CAACCTCGCCCTGTCAAACTC	3536–3556	89	G	5′-TACGATGGGATGATATTTGGGGAC	4138–4161	Present study
90	G	5′-GACCCGGCAAGGCACACTAT	3983–4002	91	NV	5′-TTTCTCCGCTCGTCCTCCG	4591–4609	Present study

strand RNA concentrations were adjusted to 200 ng μl^{-1}, and the RNA was aliquoted and stored at –70°C.

Positive- and negative-strand qRT-PCR/cDNA synthesis and reverse transcription

For the detection of positive-stranded RNA, we isolated 0.5–2 µg of total RNA from fish organ pools using an RNeasy Mini Kit. Reverse transcription was performed according to Purcell et al. (2006) and Chico et al. (2006). Tagged antisense primer (reverse) was used to synthesize positive-strand-based cDNA and tagged sense primer (forward) to synthesize negative-strand-based cDNA. The VHS tagged and sequence-specific PCR primers (Med Probe) are listed in Table 3.

Sequencing of isolated VHSV strains

To determine the genotype of the VHSV strains, the complete sequence of the glycoprotein (G) gene of 3 rainbow trout and 3 whitefish VHSV isolates (Table 4) were amplified in 4 separate RT-PCR reactions per isolate and the amplicons were sequenced with primers used in the PCRs. RT-PCR amplifications were performed as described in Raja-Halli et al. (2006). Primer sequences (Table 3) targeting VHSV G, matrix (M) and non-structural (NV) gene regions were based on the published genome of VHSV strain Fi13 (GenBank accession number Y18263.1; Schutze et al. 1999).

Sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems) and the reactions were run on an ABI 3100-*Avant* genetic analyser. For each sample, the individual PCR sequences were edited and assembled into contigs using Lasergene SeqMan Pro version 8.0.2 (DNASTAR). The assembled sequences were aligned with G gene sequences of selected Finnish and other VHSV isolates belonging to genotypes I–IV (Table 4) using ClustalX (Thompson et al. 1997) and the phylogenetic analysis was performed with MEGA 4.1 software (Tamura et al.

Table 4. Sequenced viral haemorrhagic septicaemia virus (VHSV) isolates from the present study and reference VHSV isolates used in the sequence comparisons

VHSV isolate	Host	Location and year of isolation	Genotype	GenBank accession number
Fi08.50RT	Rainbow trout	Finland 2008, present study	Id	MF176925
Fi06.59RT	Rainbow trout	Finland 2006, present study	Id	MF176926
Fi06.108RT	Rainbow trout	Finland 2006, present study	Id	MF176927
Fi08.22WF	Whitefish	Finland 2008, present study	Id	MF176928
Fi08.23WF	Whitefish	Finland 2008, present study	Id	MF176929
Fi08.24WF	Whitefish	Finland 2008, present study	Id	MF176930
FiA02a.01	Rainbow trout	Finland 2001	Id	AM086358
Fi19.04	Rainbow trout	Finland 2004	Id	AM086383
Hededam	Rainbow trout	Denmark 1972	I	Z93412
Fi13 F1	Rainbow trout	Germany 1998	I	Y18263
DK 1p52	Sprat	Baltic Sea 1996	II	AY546576
DK 1p53	Herring	Baltic Sea 1996	II	AY546577
FR L59X	Eel	France 1987	III	AY546618
DK 4p168	Herring	Skagerrak 1997	III	AY546582
US Makah	Coho salmon	Washington, USA, 1988	IV	U28747
JP KRRV9822	Japanese flounder	Japan 2000	IV	AB179621

2007). The percentage identities between paired nucleotide sequences were calculated with Megalign (DNASTAR).

Statistical analysis

The 95% confidence intervals for observed proportions were calculated with the EpiTools calculators (Sergeant 2016) using Jeffrey's method (Brown et al. 2001). The median prevalence estimate (%) was calculated using R (R Core Team 2016).

RESULTS

Virus detection from wild fish

A total of 1636 wild fish, representing 17 different fish species, were caught and sampled (Table 1). All samples tested negative by virus isolation but 4 samples gave a weak signal with qRT-PCR (C_t > 36). These samples were negative for VHSV positive-stranded RNA products, indicating that virus replication had not occurred in the tested fish at the time of sampling. Although no infected fish were detected, chance may have contributed to this non-detection, especially in those species with only few fish caught, as can be seen from the 95% confidence intervals that provide estimates of true prevalence ranges, assuming a perfect test (Table 1). The median prevalence estimates show that the true prevalence would be closer to zero than the upper confidence limit.

Infection trial in field conditions

A clinical outbreak of VHS was diagnosed in the rainbow trout on the farm 5 wk before the trial started, and mortalities due to VHSV occurred as long as the diseased rainbow trout population was present in the farm. All rainbow trout in the farm were moved to a nearby summer farming site 14 d after the infection trial started, and only the test cages were left on the farm. Whitefish were the only species in the infection trial infected by VHSV. The infection was detected in only 1 of the 3 parallel groups of whitefish, from samples collected on Days 10 and 14. The virus was not isolated on Day 10, but qRT-PCR gave weak positive signals in all tissue samples from this group. On Day 14, VHSV was detected from all tissues tested with all 4 methods used (Table 2). The positive result of the strand-specific qRT-PCR method proved that VHSV replicated in whitefish and was not a contamination from the environment. Additionally, a sprivivirus was isolated from all fish species in this infection trial (Table 2). Spriviviruses grow in the same cell cultures as those used for VHSV isolation and were confirmed by sequencing (Holopainen et al. 2017).

The control groups outside the VHS restriction area were all negative for VHSV and sprivivirus.

Sequencing of isolated VHS viruses

The G gene of VHSV from the farmed rainbow trout and the whitefish from the infection trial was

sequenced and compared with G gene sequences of some Finnish and other VHSV isolates belonging to genotypes I–IV (Table 4, Fig. S1 in supplement at www.int-res.com/articles/suppl/d131p187_supp.pdf). Pairwise sequence comparisons showed that the rainbow trout and whitefish isolates of the present study (GenBank accession numbers MF176925–MF176930) were most closely related to the Finnish rainbow trout genotype Id isolates from the Province of Åland isolated during the years 2001 and 2004 (Raja-Halli et al. 2006). The nucleotide identity between these isolates was 99.4–99.9%. The G gene sequence identity between VHSV isolates from rainbow trout and whitefish of the infection trial and VHSV isolates belonging to genotypes III and IV were approximately 91% and 86%, respectively. The 3 whitefish isolates from the infection trial and the rainbow trout Fi08.50RT originating from the same farm and year (2008) as the whitefish isolates were 100% identical to each other. These results indicate that whitefish in the infection trial were infected with the same VHS virus that was present in the farmed rainbow trout on the farm where the infection trial was performed.

DISCUSSION

Wild fish are not a likely source for the reappearing VHS outbreaks in the Finnish brackish-water fish farms. Although wild fish have been reported to be carriers of several different VHSV genotypes in the North Sea and the Baltic Sea (Skall et al. 2005), there are no reports indicating that VHSV Id in wild fish is connected with the clinical disease in farmed fish. The number of different fish species in close vicinity of the fish farms in the present study was high, but the number of individuals of each fish species varied from only a few individuals for species such as pike or sea trout to thousands for species such as perch, roach or three-spined sticklebacks. In our study, the observed prevalence of VHSV was zero in 17 different tested wild fish species that were screened during several years in the vicinity of 2 infected fish farms. The results indicated that if the screening missed the infection in these species caught in quite high numbers (see Table 1), the prevalence would have been no more than 4% (the upper limit of the 95% confidence limit is lower than that). The sample size was low for defining the possible prevalence range of some species and the results are inconclusive. The fish species that were caught in small numbers are species for which presence on the farm or in the vicinity was apparently low, and they comprise less than 5% of all fish analysed. The low numbers of these species on the fish farm are likely representative of the actual numbers present, as fish farms are not the normal habitat for these species. Organ pools from fish species caught in low numbers contained organs from fewer individuals than those from species caught in high numbers, reducing the possible effect of virus dilution.

There are several facts that support our result of the minor role of wild fish in VHSV transmission. The farms where the wild fish were caught experienced several outbreaks of VHS in their fish during the study. If the wild fish had a major role, one would expect to find clear positive signals with qRT-PCR from the tested wild fish as VHSV was present in the environment on several of the sampling occasions. In Finland, there have been 2 other VHS restriction areas, in similar farms producing rainbow trout, where the viruses were successfully eradicated at the first attempt. These areas have the same kind of wild fish populations as the farms in the study area in the Province of Åland. It is possible that some of the tested fish species could be transient carriers of the virus and therefore were not caught using the screening method. More studies on the prevalence of VHS in wild fish are needed, as all fishing methods have species and size selectivities that may bias the results.

Two wild fish species, roach and perch, which are among the most common species living in the vicinity of brackish-water fish farms, were also exposed to a natural VHSV infection under stressful conditions in small cages, but no VHSV was isolated from these fish. On the contrary, farmed whitefish kept in the same conditions as roach and perch were infected by VHSV Id. The virus was detected for a short period, but no mortalities were recorded. It is possible that whitefish are transient carriers of VHSV as the virus was not detected in the whitefish groups after the infected rainbow trout were moved away from the farm. According to the Finnish authorities, VHSV has only been isolated once from farmed whitefish in the VHS restriction area (P. Vennerström unpubl. data).

A sprivivirus was isolated from all tested fish species in the infection trial, but it was not associated with mortalities. The skin of the fish infected with sprivivirus turned reddish in colour as a result of congestion in the skin, but internal pathology was not detected in these fish. These findings were not studied further in this trial. Sprivivirus has occasionally been isolated from farmed sea trout in the study area in connection with bacterial fish diseases during the summertime, when water temperatures are greater

than 15°C (R. Holopainen, pers. comm.). *Sprivivirus* seems to be an endemic virus in the study area and it is not clear whether this virus could be a predisposing factor of the VHSV outbreaks or influence on screening of VHSV. Spriviviruses have not been reported from the other 2 successfully eradicated restriction zones of VHS.

Whitefish could be a potential source of the recurring VHS outbreaks in the VHS restriction area, as they were either farmed in the same farms as rainbow trout or have close contact with these farms. Whitefish are brought as juveniles to the farms and are farmed for 1 to 2 yr in the brackish-water fish farms. Whitefish is a native species of the Baltic Sea, where VHSV is endemic. Rainbow trout, on the other hand, is not a native species and may therefore be more susceptible to VHSV infection than native Baltic fish species. This is supported by the fact that mortality caused by VHSV Id in Finnish whitefish has never been reported, although whitefish are also screened repeatedly for VHSV according to regulations. In our study, no wild whitefish were caught during the wild fish screening, but according to our infection trial with farmed juvenile whitefish, we found that they were infected by VHSV and that replication of the virus occurred for a short time period in their organs. It seems that whitefish are not sensitive to VHSV Id infections and that they may clear out the virus infection without developing notable clinical signs. This result is also supported by the fact that no mortalities caused by VHSV in farmed white fish have been reported during more than 10 yr of disease monitoring of farmed whitefish in the VHSV-infected fish farms. Since whitefish may be infected by VHSV Id and the virus can replicate in this fish species, whitefish kept close to VHSV-positive rainbow trout populations may give the virus an opportunity to jump to whitefish and survive longer in the area. It also gives the virus the opportunity to adapt and become more virulent to whitefish, and perhaps even to other native species in the Baltic Sea. Danish scientists have reported that European whitefish are susceptible to infection with some rainbow-trout-adapted VHSV types (Skall et al. 2004). However, VHSV was not detected in wild-caught whitefish in a river system close to VHS-positive fish farms (Skall et al. 2004).

On the west coast of Sweden, VHSV was isolated from herring caught close to rainbow trout farms. On these farms, VHSV genotype Ib had caused several outbreaks on the farms, although the farms had been repeatedly emptied of fish, including removal and disinfection of all farming equipment. The herring isolates were almost identical to the genotype that was causing VHS in the farmed rainbow trout. It was assumed that the herring on their spawning migration brought the infection repeatedly to the farm and eventually made rainbow trout farming in that area impossible (Nordblom & Norell 2000, Jansson & Vennerström 2014). Another example of wild fish being the source of VHS outbreaks is from Norway, where VHSV genotype III was isolated from 4 sea-reared rainbow trout farms in Storfjorden in 2007 (Dale et al. 2009). Although VHSV III had earlier been isolated from several wild marine fish species and infection trials had proven rainbow trout not to be sensitive to genotype III strains, mortality was recorded in these outbreaks. Sequence analyses of the isolated VHSV III strain in Norway revealed it to be unique. According to infection trials, rainbow trout was reported to be susceptible to this new genotype III strain (Dale et al. 2009). The source of the infection is not clear, but these VHSV-positive farms had close connections to cod *Gadus morhua* and saithe *Pollachius virens* farming, where raw processed fish of marine origin are used for feed. Containers of dead farmed rainbow trout, cod and saithe from other sites were stored close to the primary infected site (Dale et al. 2009).

CONCLUSIONS

According to our study, wild fish living freely in the fish farming area do not seem to threaten the farmed fish with respect to VHSV Id infection in Finland. Farmed whitefish as a native species was a likely source of the recurring VHS outbreaks in Finnish brackish-water food fish farms, as they were infected but cleared out the infection. Wild fish may function as carriers between closely situated farms. Therefore, it is important to perform stamping out of infected fish farms and decrease the infection pressure and adaptation possibilities in other fish species.

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Presence of viral haemorrhagic septicaemia virus (VHSV) in the environment of virus-contaminated fish farms and processing plants

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ABSTRACT: After the first outbreak of viral haemorrhagic septicaemia virus (VHSV) in Finnish brackish water rainbow trout *Oncorhynchus mykiss* farms, infection spread rapidly between the farms. The infrastructure of fish farming did not take into account spreading of infectious fish diseases. To show the presence of VHSV in the environment, we tested seawater, sediment and wild blue mussels *Mytilus edulis* from VHSV-infected fish farms, and liquid waste from a processing plant that handled infected rainbow trout. Additionally, blue mussels were bath-challenged with VHSV (exposed to cultivated virus or naturally infected rainbow trout). To detect VHSV, virus isolation in cell culture and real-time reverse transcriptase polymerase chain reaction (qRT-PCR) were used. The virus or viral RNA was detected in sea water and in liquid waste from processing plants during wintertime when water temperature is close to 0°C and sunlight is sparse. VHSV did not appear to replicate in blue mussels in our study. Therefore, blue mussels were not considered relevant carriers of VHSV. However, traces of viral RNA were detected up to 29 d post challenge in mussels. Contact with water from processing plants handling VHSV-infected fish populations increases the risk of the disease spreading to susceptible fish populations, especially during cold and dark times of the year.

KEY WORDS: Viral haemorrhagic septicaemia virus · Environment · Sea water · Liquid waste · Blue mussels · Processing plant · UV-light

1. INTRODUCTION

In 2000, in the Province of Åland, Baltic Sea, Finland, several brackish water fish farms producing rainbow trout *Oncorhynchus mykiss* for consumption were infected by viral haemorrhagic septicaemia virus genotype Id (VHSV Id) (Raja-Halli et al. 2006). The virus spread rapidly to almost all fish farms, resulting in the entire province being declared a restriction area in 2001 (Raja-Halli et al. 2006).

VHSV belongs to the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Walker et al. 2000). It is a single-stranded enveloped RNA virus that is categorized into 4 genotypes (I–IV), of which I and IV have several sublineages (Ia–Ie, IVa–IVc) (Snow et al. 1999, Einer-Jensen et al. 2005, Elsayed et al. 2006, Ammayappan & Vakharia 2009, Pierce & Stepien 2012). VHSV is shed in the water via fish urine and reproductive fluids, and virus has been shown in subclinical and clinically affected rainbow trout as well as survivors of the disease (Wolf 1988, Oidtmann et al. 2011). VHSV-positive fish farms and liquid waste from processing plants handling VHSV-positive fish are considered a risk to susceptible fish species if released into the environment (Hervé-Claude et al. 2008, Bain et al. 2010, VHSV Expert Panel and Work-

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ing Group 2010, Phelps et al. 2012, Pearce et al. 2014, Oidtmann et al. 2018).

Blue mussels *Mytilus edulis* are common inhabitants of brackish water fish farms in the Baltic Sea. The mussels attach to farm equipment such as anchor ropes and supporting framework where they feed by filtering particles from the water. The seabed beneath the net pens is also covered by mussels that cannot be removed when a fish farm is fallowed due to notifiable fish diseases. The role of shellfish used for consumption as carriers of viral pathogens of human origin such as noroviruses, enteroviruses and hepatitis A virus is well described (Richards 1985, 1988, Power & Collins 1989, Kingsley & Richards 2003). The role of mussels as transmitters of fish pathogens is not well known. There are indications that infectious pancreatic necrosis virus (IPNV) and other aquatic birnaviruses may persist in mussel tissue (Mortensen et al. 1992, Rivas et al. 1993). Furthermore, a challenge study showed that blue mussels were able to transfer IPNV to challenged Atlantic smolts (Molloy et al. 2013). IPNV is a non-enveloped birnavirus that is resistant to physico-chemical factors (Bovo et al. 2005). On the other hand, infectious salmon anaemia virus (ISAV), which is an enveloped virus like VHSV, did not persist in mussels after the source of infection was removed and thus mussels are not considered as potential transmitters of ISAV (Skår & Mortensen 2007). In another challenge of blue mussels with ISAV, viral RNA was detected in all samples from the 144 h challenge, but all samples were negative by culture analysis (Molloy et al. 2014). There are no reports on whether blue mussels could act as transmitters of VHSV or shed the virus. Survival of VHSV outside the host depends on physico-chemical conditions in the environment (Bovo et al. 2005). In cold water (4°C), VHSV can survive for a few days in natural fresh water or seawater and up to a year in filtered fresh water (Parry & Dixon 1997, Hawley & Garver 2008). In warm temperatures (20°C), VHSV is less stable (Hawley & Garver 2008). Fresh water seems to be more favourable for virus survival than seawater (Hawley & Garver 2008). Rhabdoviruses such as VHSV and infectious haematopoietic necrosis virus are sensitive to ultraviolet (UV) irradiation (Øye & Rimstad 2001, Yoshimizu et al. 2005, Afonso et al. 2012).

Attempts to eradicate VHS from the fish farms in the restriction area in the Province of Åland failed several times (Raja-Halli et al. 2006). VHS-positive fish farms were emptied of fish, and farming equipment was removed, washed and disinfected according to instructions from the authorities. After repopulation of fallowed (>8 wk) farms with fish from a VHSV-free area, new infections were detected as early as 2 wk after repopulation (our own observations). In earlier studies performed in this restriction area, we described different surveillance procedures and diagnostic methods to screen for VHSV-infected fish populations (virus isolation in cell culture and real-time reverse transcriptase polymerase chain reaction [qRT-PCR] and serology to detect antibodies against VHSV). We found active surveillance performed by the fish farmers, whenever there were signs of a possible fish disease, to be more effective than passive surveillance. The results of the qRT-PCR method corresponded well with the results from the parallel testing of the same samples with virus isolation in cell culture (Vennerström et al. 2017). We screened wild fish living in the vicinity of VHSV-infected fish farms in this restriction area for VHSV during 4 years, but wild fish were not found to be relevant carriers of this virus (Vennerström et al. 2018). On the other hand, whitefish *Coregonus lavaretus* that were cultured in the same farms or close to VHSV-infected rainbow trout populations were probable disease transmitters, as they were found to be infected by VHSV without observed mortality in an infection trial (Vennerström et al. 2018).

The infrastructure of fish farming in the study area did not consider spreading of infectious diseases. Processing plants were important to fish farming practices, and contacts between fish farms and processing plants occurred daily. Contacts between infected fish populations were also common during daily servicing of fish farms by personnel and boats. In the present study, we looked for possible reservoirs of the virus in the environment surrounding the fish farms and processing plants, such as wild mussels, sediment, seawater from VHSV-infected farms and liquid waste from plants processing VHSV-positive fish populations. No studies have addressed these issues concerning VHSV genotype Id in brackish water fish farms in a VHSV restriction area, and no studies of the persistence of VHSV in mussels have been reported. To address whether blue mussels could be carriers of VHSV by protecting the virus from environmental factors such as UV light, we tested wild mussels living in VHSV-infected fish farms for VHSV and performed 2 infection trials with mussels in VHSV-contaminated aquarium water.

Information on the source of VHSV in the environment was needed to plan eradication measures and point out to farmers possible sources of infection and the importance in changing the infrastructure to consider infectious fish diseases.

2. MATERIALS AND METHODS

2.1. Collection of seawater, sediment and liquid waste samples

In April and May 2008, as well as in January and March 2009, seawater samples were collected from the close vicinity (<1 m) of net pens in 2 fish farms: Farm A owned by Company 1 and Farm B owned by Company 2. Both companies produced rainbow trout for human consumption in the Baltic Sea on the southwest coast of Finland. The processing plant of Company 1 was situated next to Farm A. The processing plant of Company 2 was situated >5 km from Farm B, but whitefish *Coregonus lavaretus* were farmed next to the processing plant during this study. Both Farms A and B had rainbow trout populations experiencing a clinical VHS outbreak at the time of sampling. Seawater samples were also collected at the loading dock of the fish processing plant of Company 2 at the time VHSV-positive rainbow trout were processed; however, during sampling in March 2009, only whitefish were processed at this plant. Water temperatures were 4°C in April 2008, 7–10°C in May 2008 and approximately 0°C in January and March 2009. Water samples of 5 l were collected from the surface and from 2 m depth. During April and May, altogether 40 samples were collected on 3 occasions. Sediment was collected from the seabed beneath net pens of Farm A with an Ekman grab sampler. Liquid waste was collected in January and March 2009 from different parts of the processing plant of Company 2: the carbon dioxide stunning basin, bleeding basin, kidney remover and liquid waste drain before and after final decontamination treatment. All water samples were protected from sunlight, kept cool during transport and storage, processed and tested in the Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki. Sampling sites and the number of water, sediment and liquid waste samples collected are shown in Table 1.

Table 1. Results of testing for viral haemorrhagic septicaemia virus (VHSV) in seawater, sediment and liquid waste water from 2 VHSV-positive fish farms (A and B) and a plant that processed VHSV-positive fish. CPE: cytopathic effect; N: number; nd: not done; pos: VHS-positive samples; PP: processing plant of Company 2; qRT-PCR: direct real-time reverse transcriptase polymerase chain reaction; water filtering: water samples were filtered before testing with qRT-PCR

Farm	Sampling date	Water temp. (°C)	Sample type	Sample origin	N samples (pooled for qRT-PCR)	Water filtering	Virus isolation N CPE pos / N samples	qRT-PCR	
								N pos / N CPE pos cell culture	N water samples pos / N tested
Company 1									
A	April–May 2008	4	Seawater	Net pens with VHSV-positive trout	21	Yes	nd	nd	1/21
A	April 2008	4	Sediment	Under net pens VHSV-positive trout	10		nd	nd	0/10
Company 2									
B	May 2008	10	Seawater	Net pens with VHSV-positive trout	19	Yes	nd	nd	0/19
PP	January 2009	2	Seawater	Net pens with VHSV-positive trout	3(1)	Yes	0/3	nd	1/1
			Seawater	Loading dock of slaughterhouse	3(1)	Yes	0/3	nd	1/1
PP	January 2009	2	Liquid waste	Stunning basin	3		2/3	2/2	3/3
			Liquid waste	Bleeding basin	3		1/3	1/1	3/3
			Liquid waste	Kidney remover	2		2/2	2/2	2/2
			Liquid waste	Drain before disinfecting	3		3/3	3/3	3/3
			Liquid waste	Drain after disinfecting	3		0/3	nd	0/3
PP	March 2009	0	Seawater	Loading dock of slaughterhouse	2	Yes	0/2	nd	2/2
PP	March 2009	0	Liquid waste	Stunning basin	2(1)		1/2	1/1	1/1
			Liquid waste	Bleeding basin	2(1)		2/2	2/2	0/1
			Liquid waste	Kidney remover	2(1)		2/2	2/2	1/1
			Liquid waste	Drain before disinfecting	2(1)		0/2	nd	0/1

2.2. Wild blue mussels for virological examination

Blue mussels living on anchor ropes in Farms A and B and on a third fish farm (control Farm C) were tested for VHSV. The sampling scheme is presented in Table 2. Farm C also produces rainbow trout for consumption in net pens in the Baltic Sea but is situated outside of the VHS restriction area where VHSV has been screened for since 1995 but never reported. Mussels from control Farm C were tested in May 2007 at a water temperature of 9°C. Mussels from all farms were transported in a cooled transport box on moist paper to the Finnish Food Safety Authority (Evira, now named the Finnish Food Authority) in Helsinki to be tested for VHSV.

2.3. Collection of mussels for bath challenge studies

Mussels (length ca. 3 cm) for 2 different bath challenges were collected from the anchor ropes of control Farm C and transported to the laboratory at Evira (first bath challenge) and to a VHSV-positive fish farm (second bath challenge). From the control farm, mussel samples were also collected for VHSV testing.

2.3.1. Bath challenge of mussels with VHSV

Three test aquariums (I, II, III), 2 l each, were kept in a refrigerated dark room at 5°C. Seawater for the aquariums was transported in plastic canisters from control Farm C. Sixty mussels were selected and placed arbitrarily in each aquarium. The aquariums were aerated, and the water was changed daily to imitate the natural water currents on the farm where water is changing continuously. The mussels attached to the surface of the aquarium and started filtering water after a few hours. The bath challenge was started the day after transfer. Just before the challenge started, the water from all 3 aquariums (I–III) was removed, the mussels were rinsed 3 times with seawater, and the aquariums were each filled with 2 l of seawater. The rinse was performed to make the environment as free from faeces excreted by the mussels as possible before adding 5 ml of VHSV strain Fika422, genotype Id (GenBank accession no. AY546615; Einer-Jensen et al. 2004), virus titre 10^7 $TCID_{50}$ in each aquarium. The virus had been cultivated in bluegill fry fibroblast (BF-2) cells (Wolf et al. 1966) growing in Eagle's minimal essential medium (MEM) at 16°C until complete destruction of the cell monolayer. The virus-containing medium was added to 2 test aquariums (I and II). Aquarium III was used as a negative control, and 5 ml of sterile MEM were added to this aquarium. The control aquarium was treated and sampled in the same way as the 2 test aquariums. Two different challenge times were used: 6 h for Aquarium I and 24 h for Aquarium II. Before the challenge was terminated, 10 live mussels from each treated aquarium were collected to be tested for VHSV. The vitality of the collected mussels was determined by evaluating their ability to filter water and to close their shell when experiencing physical contact. Pieces of hepatopancreas of 5 mussels were sampled and pooled in 9 volumes of MEM (proportion of tissue to MEM 1:10); the pooled samples were used in virological examinations. The aquarium water was replaced at the end of each sampling. The remaining mussels in the aquarium were rinsed 3 times with fresh seawater before the aquarium was refilled with new seawater. Samples were collected arbitrarily at intervals described in Table 3. All waste water was poured into plastic canisters and treated with Virkon™ S according to the manufacturer's instructions before being poured into the disinfection tank that heated waste water to 127°C for 60 min. The outer surfaces of the aquariums and other equip-

Table 2. Results of testing for viral haemorrhagic septicaemia virus (VHSV) in the hepatopancreas of wild blue mussels from 2 VHS-positive fish farms (A and B) farming rainbow trout for consumption in the Province of Åland, Finland, and from a similar farm situated in a VHS-free zone on the west coast of continental Finland used as a control farm (C). N: number; nd: not done; qRT-PCR: real time reverse transcriptase polymerase chain reaction

Farm	Sampling date	N mussels	N pools	N pools positive / N pools tested	
				Cell culture	qRT-PCR
Company 1					
A	April 2006	13	7	0/7	nd
A	May 2006	10	10	0/10	1[a]/10
A	November 2006	100	20	0/20	0/20
Company 2					
B	May 2006	10	5	0/5	nd
B	June 2006	50	10	0/10	0/10
Control farm					
C	May 2007	10	10	0/10	0/10
Total		193	62	0/62	1[a]/50

[a]Weak signal with threshold cycle cut-off >36

ment used were disinfected with 70% ethanol, and the surface of the aquarium table and the cool-room floor were treated with Virkon™ S every time the water was changed. The aquariums were covered daily with new plastic sheets to avoid cross contamination of the virus between study groups and contamination by disinfectants.

2.3.2. Bath challenge using VHSV-infected rainbow trout

The second bath challenge was performed at Farm A during a clinical outbreak of VHS. Mussels (n = 200) collected from Farm C were divided into 2 groups of 100 mussels each (Group I and Group II) and placed arbitrarily into 2 different aerated tubs each filled with 10 l of seawater from Farm A. The tubs were kept in a refrigerated dark room at 8°C. Four rainbow trout of approximately 1 kg each, with symptoms typical of acute septic infection, i.e. dark skin colour and exophthalmia, were collected from the farm and transferred into the tubs, 2 fish in each. The first 2 fish were held together with Group I for 10 min and the other 2 fish were kept with Group II for 20 min. Due to ethical issues, the diseased rainbow trout were kept in the tubs for as little time as possible. To determine whether the fish used for the infection trial were infected by VHSV, the fish were euthanized and necropsied immediately after the end of exposure. Samples from the spleen, anterior kidney and heart were examined individually by cell culture and ELISA for fish viruses according to Commission Decision 2001/183/EC (EC 2001). After the fish were removed, the exposure of the mussels to the water, now presumably contaminated with VHSV, continued for an additional 4 h. The water temperature in the tub was 5°C at the beginning and 8°C at the end of the exposure. The mussels started to filter water a few minutes post transfer. At the end of the exposure, the mussels were still alive, as they reacted to physical contact by closing their shells. Immediately after the end of exposure, 10 mussels from different parts of each tub were non-randomly collected and sampled. These samples were transported on ice to the laboratory and further processed the next day. The remaining live mussels were transported in a cool box on wet paper to the laboratory where they were placed into 2 aquariums (2 l each;

Table 3. Viral haemorrhagic septicaemia virus (VHSV) isolations and real-time reverse transcriptase polymerase chain reaction (qRT-PCR) results from 2 bath challenges of blue mussels with VHSV grown in cell culture and VHSV from infected rainbow trout. In both trials, Aquarium I and II are test aquariums and Aquarium III is a negative control aquarium in which all results were negative and are not shown in the table. N: number; nd: not done; CI: confidence interval

Time of sampling	Aquarium	N samples positive / N samples tested		
		Mussel hepatopancreas		Aquarium water
		Virus isolation in cell culture	qRT-PCR	qRT-PCR
Bath challenge with VHSV				
0 (before challenge)	I, II, III	0/5	nd	nd
6 h (at end of challenge)	I	5/5	5/5	nd
1 d	I	0/5	3/5	nd
1 d (at end of challenge)	II	0/5	4/5	nd
2 d	I	0/5	1/5	nd
2 d	II	0/5	2/5	nd
3 d	I	0/5	0/5	nd
3 d	II	0/5	4/5	nd
6 d	I	0/5	3/5	nd
6 d	II	0/5	2/5	nd
Total (N, %, 95% CI)		5/50 (10, 4–21)	24/45 (53, 39–67)	
Bath challenge with VHSV-infected rainbow trout				
0 (before challenge)	I, II, III	0/3	0/3	0/1
At end of 10 min challenge	I	0/2	2/2	2/2
At end of 20 min challenge	II	0/2	1/2	2/2
1 d	I	0/2	1/2	1[a]/1
	II	0/2	1[a]/2	1[a]/1
2 d	I	0/2	2/2	1[a]/1
	II	0/2	0/2	1[a]/1
3 d	I	0/2	1/2	0/1
	II	0/2	0/2	0/1
4 d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
6 d	I	0/2	1[a]/2	0/1
	II	0/2	1[a]/2	0/1
8 d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
11 d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
14 d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
22 d	I	0/2	1[a]/2	0/1
	II	0/2	0/2	0/1
27 d	I	0/2	0/2	0/1
	II	0/2	0/2	0/1
29 d	I	0/2	1[a]/2	0/1
	II	0/2	0/2	0/1
Total (N, %, 95% CI)		0/51 (0, 0–7)	7/51 (14, 7–26)	4/27 (15, 6–32)

[a]Weak signal with threshold cycle cut-off >36

Aquarium I and Aquarium II) the day after exposure. The aquarium water that was used for the infection trial was transported from control Farm C. One aquarium with 100 mussels from Farm C was prepared as the control (Aquarium III). Five filtering mussels from each aquarium and 1 l of aquarium water were collected before the water was changed and examined for the presence of VHSV on Days 1–4, 6, 8, 11, 14, 22, 27 and 29 (Table 3). The treatment of the aquariums and the facilities to avoid viral contamination was the same as that described for the first bath challenge.

2.4. Examination of samples from mussels, seawater and liquid waste for VHSV by virus isolation

The samples from seawater, liquid waste and hepatopancreas were processed according to standard virological procedures. The hepatopancreas of a maximum of 5 mussels was pooled in 9 volumes of MEM, homogenized and centrifuged at 4000 × *g* (20 min at 4°C). The seawater and liquid waste samples were diluted similarly but not homogenized. All samples were kept on ice during the process. Supernatants from the organ homogenate, diluted water samples and liquid waste were collected, and 150 µl were inoculated into 24-well tissue culture plates with monolayers of 2 different cell lines: bluegill fry fibroblast BF-2 cells (Wolf et al. 1966) and epithelioma papulosum cyprinid cells (Fijan et al. 1983, Olesen & Vestergård Jørgensen 1992). The remaining supernatant was frozen to –80°C for later examination using qRT-PCR. The inoculated cells were cultivated for 2 consecutive passages for a total of 14 d. Cell cultures with cytopathic effects were collected and frozen for later confirmation of the presence of VHSV using qRT-PCR (Vennerström et al. 2017). Due to technical problems, virus isolation was not performed from seawater and liquid waste samples taken in April and May 2008.

2.5. Treatment of samples from seawater, aquarium water, liquid waste and sediment

Water samples were treated with methods described by Maunula et al. (2012) with some modifications. In general, 5 l of the seawater samples were prefiltered through a Waterra® filter (FHT-700) (Powell et al. 2000), but in some cases, only 1 or 3 l could pass through the filter. Filtering was continued through a GF/F membrane (Whatman International). Virus particles were eluted from the Waterra filter using 50 ml of 50 mM glycine-3% beef extract (pH 9.5) and from the GF/F membrane with 1 ml AVL lysis buffer (Qiagen) after shaking for 10 min at room temperature. Both eluates were subjected to RNA extraction with a Viral RNA Mini Kit (Qiagen). Aquarium water from the infection trials and the liquid waste samples were not filtered. For determining the presence of VHSV, 140 µl of each sample were collected for RNA extraction with a Qiagen Viral RNA Mini Kit. Sediment samples were diluted by taking 5 g of each sample and adding 1 ml of phosphate-buffered saline. Suspensions were briefly stirred, and 200 µl of the liquid were taken for RNA extraction, which was performed using a Nuclisens magnetic extraction kit (Biomérieux). RNA was analysed using qRT-PCR both undiluted and in 1:10 dilution (RNase-free water).

2.6. qRT-PCR

The supernatants from the hepatopancreas–MEM suspension that was prepared for virus isolation, supernatants from the cell culture showing a cytopathic effect and the sediment and water samples were examined for the presence of VHSV using a qRT-PCR method published earlier by Vennerström et al. (2017). The qRT-PCR method was compared to virus isolation in cell culture and correlated well with the virus isolation results (kappa value = 0.877, sensitivity = 1, specificity = 0.959; Vennerström et al. 2017). Briefly, qRT-PCR was performed with a QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The primers and the probe (MedProbe) for the qRT-PCR were designed according to the VHSV nucleocapsid gene sequence from GenBank, accession no. D00687, after Chico et al. (2006) (Table 3). A threshold cycle (C_t) cut-off of 36 (<30 copies) was used in the analysis as estimated in our earlier study (Vennerström et al. 2017). However, results with $C_t > 36$ (showing a sigmoidal amplification curve) are considered as possible traces of VHSV RNA. The amplification efficiency of the qPCR reaction of a standard curve based on the slope (–3.44) was 96.8%.

2.7. Statistical analyses

Due to small numbers of samples, data were only described. We used 95% confidence intervals (CI) for comparison of percentages. They were calculated

using Epitools (Sergeant 2019) with the Wilson method (Brown et al. 2001).

3. RESULTS

3.1. Occurrence of VHSV in environmental samples from fish farms and a processing plant

The sampling scheme, methods and results from the testing of samples for VHSV are presented in Table 1.

3.1.1. Seawater

Only 1 sample taken from seawater at Farm A in April 2008 (4°C) gave a weak positive reaction (95% CI: 0.8–23) when tested for VHSV RNA using qRT-PCR (Table 1). All seawater samples collected in May 2008 (4–10°C) from Farm B were negative (95% CI: 0–17) for VHSV RNA. Virus isolation was not performed from any samples taken in April and May 2008. All samples that were collected in January and March 2009 (0–2°C) were positive (95% CI: 30–95) for VHSV RNA using qRT-PCR after filtering, but no virus could be isolated from the same samples. No difference was noticed between the samples taken from the surface or from 2 m depth.

3.1.2. Sediment from the sea bed

All collected samples from the sediment beneath the fish farms were negative (95% CI: 0–28) for VHSV RNA using qRT-PCR.

3.1.3. Liquid waste

All liquid waste samples taken in January 2009 (2°C) from the processing plant before liquid waste disinfection were positive for VHSV RNA using qRT-PCR (95% CI: 74–100), and 73% of the samples tested by cell culture (95% CI: 43–90) were positive for VHSV (Table 1). No virus could be detected by either method in samples taken after final disinfection of the liquid waste. In March 2009 (0°C) at the second sampling time, when only whitefish were processed, 50% of the samples tested positive using qRT-PCR (95% CI: 15–85), and VHSV was isolated from 63% of the samples (95% CI: 31–86). The liquid waste disinfection system was not running at the time of the second sampling, and therefore disinfected effluent could not be collected for testing.

3.2. Occurrence of VHSV in wild mussels

VHSV was not isolated from the 62 pools of 193 blue mussels collected from fish farms (Table 2) that were tested (95% CI: 0–6). Altogether, 50 pools of organ samples from blue mussels were tested using qRT-PCR and were found to be negative (95% CI: 0–7) for VHSV RNA, except in May 2006, when 1 sample from Farm A gave a weak signal ($C_t > 36$).

3.3. Infection trials with blue mussels

3.3.1. Bath challenge of blue mussels with VHSV

All tests from mussel samples taken before the challenge started and from control Aquarium III were negative (Table 3). VHSV was isolated only once in this bath challenge performed with VHSV. The isolation was made from the hepatopancreas at the end of a 6 h challenge in Aquarium I (Table 3). No virus was isolated from Aquarium II mussels (1 d challenge) on any occasion. However, qRT-PCR gave positive signals for VHSV RNA in both Aquariums I and II throughout the follow-up period of 6 d.

3.3.2. Bath challenge of blue mussels with VHSV-infected rainbow trout

The 4 rainbow trout used to challenge mussels in the infection trial showed typical signs of a septic infection at necropsy: dark skin colour, exophthalmia, reddish fluid in the abdominal cavity and petechiae in the skin, visceral adipose tissue, liver and muscle tissue. VHSV was isolated from all 4 individually tested rainbow trout when analysed after the challenge. Genotype was not determined, but VHSV genotype Id had been isolated from the same fish population 3 d earlier (Vennerström et al. 2017). Hepatopancreas samples of the blue mussels in both Aquarium I and II were VHSV negative in the cell culture throughout the follow-up period of 29 d (Table 3). qRT-PCR gave clearly positive results in Aquarium I up to 3 d post infection and in Aquarium II at the end of the 20 min challenge. In addition, traces of viral RNA ($C_t > 36$) were detected several times in the mussels throughout the follow-up period until Day 29 in Aquarium I and Day 6 in Aquarium II. In contrast, water samples that were analysed for VHSV RNA showed a weak signal for only 2 d in both groups. All samples from the control (Aquarium III) were negative by cell culture and qRT-PCR.

4. DISCUSSION

We found that in seawater close to the net pens with VHS diseased rainbow trout populations and next to loading docks of processing plants handling VHSV-positive fish, VHSV was more frequently detected at cold water temperatures during wintertime than in spring. Water temperature in the study area was close to 0°C in January–March and 4–10°C in April–May. Daylight is only 6 h in January but increases to 14–16 h in April–May (Nordlund 2008, Cornwall et al. 2020). The low amount of UV radiation in wintertime (Finnish Meteorological Institute 2019) in the study area together with short daylight hours and cold water temperature seems to be favourable for virus survival. The result is consistent with previous studies where VHSV was reported to be sensitive to UV light and survive longer in cold water temperatures than in warm (Ahne 1982, Parry & Dixon 1997, Øye & Rimstad 2001, Yoshimizu et al. 2005, Hawley & Garver 2008, Afonso et al. 2012).

The difference in temperature between winter and spring was not so high that temperature could be considered of high importance alone. According to the literature, there are indications that virus survival could be decreased in an environment with bacteria- and virus-inhibiting compounds (Mori et al. 2002, Bovo et al. 2005). Extremely low water temperatures could have a negative impact on the number of bacteria-, algae- and virus-inhibiting compounds in the water and therefore give VHSV more favourable conditions than in warmer water.

Liquid waste samples from the processing plant collected in March 2009 were positive for VHSV RNA, although only clinically healthy whitefish had been processed at the time. Whitefish were not sampled in this study, but in previous studies, we noticed that although whitefish are not easily infected with VHSV genotype Id, some fish in the population may get infected and virus replication occurs (Vennerström et al. 2018). The processed whitefish were farmed next to the processing plant where VHSV-positive rainbow trout had been processed earlier the same year. It is possible that whitefish may have been infected by VHSV from that processing plant. Another possibility for this virus-positive finding is that the processing line was highly contaminated by VHSV RNA from infected rainbow trout processed earlier. According to our study, it is possible that processing plants handling VHSV-positive fish and the surrounding environment are contaminated with the virus, especially in winter. For this reason, any contact between processing plants and farmed susceptible fish populations should be avoided, especially during the coldest and darkest time of the year. This statement is also supported by a study conducted by Oidtmann et al. (2011), who found high amounts of VHSV Ia in both sub-clinically affected and survivors of a VHSV-infected rainbow trout population. They also suggested that processed fish from an infected population and effluent from the processing plant could pose a significant risk for spreading virus.

Based on the results of our studies on blue mussels, it can be assumed that VHSV is not able to replicate in blue mussels. This was shown by taking samples from the hepatopancreas of mussels living in VHSV-infected fish farms and by 2 different infection trials with VHSV. The challenges were performed with 2 different methods, but the result was the same regardless of the method used. In our challenge studies, VHSV RNA was detected in aquarium water only during the bath challenge, but somewhat longer in the samples taken from mussels. This difference could have been due to the frequent water changes in the test aquariums in order to give the mussels as good conditions in the aquarium as possible. The finding of viral RNA in mussels could also indicate that they may serve as a physical attachment surface for VHSV, protecting the virus from environmental effects by providing a cleaner environment. VHSV is an enveloped virus that is not as resistant to environmental effects as birnaviruses, e.g. IPNV, that are non-enveloped and have been found in free-living molluscs (Mortensen et al. 1992, Rivas et al. 1993, Bovo et al. 2005). Molloy et al. (2013) showed that IPNV could be transferred by blue mussels to Atlantic salmon *Salmo salar*.

Sampling of blue mussels for conducting virology is demanding, as it is practically impossible to obtain samples without contaminating them with virus that could exist in the water in which they are living. It is also not possible to disinfect the inside of the shell without contaminating the internal organs with disinfectants that would interfere with virus isolation and give false negative results. We found parallel sampling of both hepatopancreas of challenged mussels and their aquarium water to be quite reliable in testing the role of blue mussels in preserving VHSV.

Replication of VHSV in mussels was unlikely, since if increased secretion of the virus had occurred in the mussels, one would expect the virus load in the aquarium water to increase as well. The unlikely role of blue mussels being carriers of VHSV in our study could also explain why VHS was successfully eradicated in 2 similar farming sites for rainbow trout on the west and south coast of Finland (Raja-Halli et al.

2006). These farms also had high densities of blue mussels in their environment. If the VHSV could replicate in mussel tissues, one would expect those eradications to have failed.

5. CONCLUSIONS

Processing plants handling VHSV-positive fish and seawater close to VHSV-positive fish populations are likely contaminated with VHSV during wintertime when daylight is sparse and temperatures are close to 0°C. Contact with contaminated facilities increases the risk of the disease spreading to susceptible fish populations. Based on our results, blue mussels may not be a relevant source of VHSV, as the virus was not shown to replicate in mussel tissues, but they could provide VHSV a physical protective environment that could prolong the survival time of the virus, although probably not for more than a few days.

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